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Neuropeptide Modulation of Spinal Pain Pathways

by

Timothy D. Moran ©

A thesis submitted to the Faculty of Graduate Studies and Research in
partial fulfillment of the
requirements for the degree of Doctor of Philosophy

Centre for Neuroscience
Edmonton, Alberta
Fall 2003

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"A mind that is stretched by a new experience can never go back to its old dimensions."

—Oliver Wendell Holmes

"On the mountains of truth you can never climb in vain: either you will reach a point higher up today, or you will be training your powers so that you will be able to climb higher tomorrow."

—Friedrich Nietzsche (1844-1900)

Abstract

This thesis examined modulation of pain signaling by spinal neuropeptides. It investigated the action of neuropeptide Y (NPY) and morphine-3β-D-glucuronide (M3G), a morphine metabolite, which may interact with spinal peptidergic mechanisms. The studies herein relate to the development of new therapeutic approaches for the treatment of neuropathic pain.

Experiments were performed using infrared-differential interference contrast (IR-DIC) videomicroscopy and whole-cell patch-clamp recording from neurons in transverse slices of adult rat lumbar spinal cord.

The main findings are: (1) NPY acted at a presynaptic Y2 receptor to attenuate excitatory postsynaptic currents (EPSCs); and (2) NPY acted at a presynaptic Y1 receptor to attenuate inhibitory postsynaptic currents (IPSCs). Postsynaptic Y1 receptors may also be involved in the effect on inhibitory synaptic transmission, but no evidence was found for involvement of a Y2 receptor in attenuation of excitatory transmission. In addition, NPY suppressed GABAergic and glycinergic IPSCs equally. The presynaptic effect of NPY on EPSCs was similar to the μ-opioid agonist, [D-ala^2,N-Met-Phe^4,Gly-ol^5]-enkephalin (DAMGO), both in terms of efficacy and site of action; NPY and μ-opioid receptors were frequently co-localized on the same presynaptic terminals. NPY also acted at postsynaptic receptors and activated an inwardly-rectifying conductance.

To examine possible interactions of M3G with spinal peptidergic mechanisms its action were compared to DAMGO and the opioid receptor-like$_1$ (ORL$_1$) agonist, nociceptin/orphanin FQ (N/OFQ). DAMGO and N/OFQ suppressed EPSCs and activated an inwardly-rectifying K^+ conductance. M3G neither mimicked nor interfered

with these peptidergic receptor mechanisms; its only effect was to suppress IPSCs in a naloxone-insensitive manner. IPSCs were also attenuated by DAMGO, but not by N/OFQ. Thus, the effect of M3G on IPSCs may contribute to the allodynia and hyperalgesia observed after intrathecal application of high doses of morphine.

Comparison of the global effects of M3G with the directed effects of NPY and µ-opioids suggests that inhibition of some, but not all, neurons in the inhibitory network of the substantia gelatinosa can lead to analgesia. In addition, because µ-opioid receptors are down-regulated after peripheral nerve injury, Y1 and Y2 receptor agonists may be especially relevant to neuropathic pain management, as their cellular effects resemble those of µ-opioid receptor agonists.

This thesis is dedicated to my wife, Katie. Without her constant love, support and belief in me this body of work would never have been completed.

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List of Abbreviations

aCSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methy-4 isoxazolepropionic acid
AP	action potential
ATP	adenosine triphosphate
ATP	adenosine triphosphate
BIBP3226	(R)-N2-(diphenlyacetyl)-N-[(4-hydroxy phenyl)methyl]D-argininamide
BIIE0246	(*S*)-N^2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6*h*)-oxodibenz[*b*,*e*]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5[4*H*)-dioxo-1,2-diphyenl-3*H*-1,2,4-triazol-4-yl]ethyl]argininamide
cAMP	cyclic adenosine-monophosphate
CCI	chronic constriction injury
CGRP	calcitonin-gene related peptide
CMR1	cold- and menthol-sensitive receptor
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
DAMGO	[D-Ala^2,N-Met-Phe^4,Gly-ol^5]-enkephalin
DL-AP5	DL-2-Amino-5-phosphonovaleric acid
DRG	dorsal root ganglia

EGTA	ethylene glycol-bis-(2-aminoethyl)-N,N,N', N'-tetraacetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
$GABA_BR$	$GABA_B$ receptor
GABA-IR	GABA-immunoreactive
GalR	galanin receptor
GIRK	G-protein-coupled inwardly-rectifying K^+ current
Glycine-IR	glycine immunoreactive
GlyR	glycine receptor
GPCR	G-protein-coupled receptor
HPLC	high performance liquid chromatography
HTM	high-threshold mechanoreceptor
HVA	high-voltage activated
HVA I_{Ca}	high-voltage activated calcium current
I_A	A-type K^+ current
$I_{Ca,N}$	N-type calcium current
I_H	H-current
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential

IR	infrared
IR-DIC	infrared-differential interference contrast
IR-NPY	immunoreactive-NPY
IR-Substance P	immunoreactive-Substance P
ISI	inter-stimulus interval
LTP	long-term potentiation
M3G	morphine-3β-D-glucuronide
M6G	morphine-6β-D-glucuronide
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
mRNA	messenger ribonucleic acid
N/OFQ	nociceptin/orphanin FQ
NA	numerical aperture
NGF	nerve growth factor
NK-1	neurokinin-1
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NST	nocistatin
OP	opioid peptide
ORL_1	opioid receptor-like$_1$
P2X	ligand-gated purinergic receptor
PAG	periaqueductal grey
PBS	phosphate-buffered saline

PKCγ	protein kinase C gamma
PNS	peripheral nervous system
PYY	peptide YY
RVM	rostral ventromedial medulla
SNL	spinal nerve ligation
SRT	spinoreticular tract
SST	somatostatin
STT	spinothalamic tract
Substance P-IR	Substance P-immunoreactivity
TrkA	receptor tyrosine kinase A
TTX	tetrodotoxin
VDCC	voltage-dependent calcium channel
VR1	vanniloid receptor

Chapter 1

General Introduction

This thesis examines aspects of the modulation of pain transmission by spinal neuropeptides. It includes an investigation of the role of neuropeptide Y (NPY) in the modulation of pain signaling in the substantia gelatinosa of the dorsal horn. The interaction of the morphine metabolite, morphine-3β-D-glucuronide (M3G) with spinal pain mechanisms is also examined, as this substance may interact with spinal peptidergic mechanisms. Investigation of the actions of spinal neuropeptides relates to an interest in developing new therapeutic approaches to the treatment of neuropathic pain.

Pain Definitions

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey, 1986; Turk & Okifuji, 2001). A variety of different types of pain is described and summarized in Table 1-1 (modified from Turk & Okifuji, 2001).

Several cellular mechanisms may account for the development of allodynia and hyperalgesia. These include central sensitization, wind-up and anatomical reorganization of the dorsal horn.

Spinal Pain Networks: Anatomy and Functional Organization

Nociceptive Sensory Fibres

Painful stimuli are detected by a specific class of nerve fibres called nociceptors that were first reported by (Zotterman, 1933). Nociceptors can be broadly classified according to their responses to different types of stimuli and the conduction velocity of their axons. Nociceptive primary afferent fibres have restricted receptive fields, are thinly myelinated or unmyelinated fibres and have conduction velocities in the Aδ (25-55m/s) and C-fibre range (< 2m/s) (Bessou & Perl, 1969; Burgess & Perl, 1967).

C-Fibres

C-fibre mechano-heat nociceptors are thought to be the unmyelinated, slowly conducting fibres (conduction velocity < 2 m/s) that were first described in detail by Perl and colleagues (Bessou & Perl, 1969). These receptors are present in the skin and are primarily polymodal nociceptors responding to intense thermal, mechanical and chemical stimuli (Dubner & Bennett, 1983; Perl, 1996). Polymodal nociceptors respond to thermal stimuli in the range of 41-49°C (Lamotte & Campbell, 1978) and a large proportion of C-fibres are capsaicin-sensitive and express the vanilloid receptor (VR1) (Caterina *et al.*, 1997; Caterina & Julius, 2001; Tominaga *et al.*, 1998). Recently, a cold- and menthol-sensitive receptor (CMR1), which may provide a cellular basis for cold sensation, has been identified and is likely present on C-fibres (McKemy *et al.*, 2002). C-fibres can also be subdivided based on histological markers. One group expresses pro-inflammatory peptides such as Substance P and calcitonin-gene related peptide (CGRP) and TrkA, the high-affinity tyrosine kinase receptor for nerve growth factor (NGF) (Snider & McMahon, 1998). These fibres project to lamina I and outer lamina II of the spinal cord (Kar & Quirion, 1995). The second group does not express Substance P, CGRP or TrkA, but does express purinergic ($P2X_3$) receptors (Snider & McMahon, 1998) and is identified by enzymes, such as fluoride-resistant acid phosphatase (Nagy & Hunt, 1982) or the lectin, IB4 (Stucky & Lewin, 1999).

Aδ-Fibres

Aδ-fibres can be functionally classified into two categories: Type I and Type II (Dubner *et al.*, 1977; Leem *et al.*, 1993). Type I Aδ fibres typically have conduction velocity in the range of 25-55 m/s and are activated by intense mechanical stimuli or by noxious heat at temperatures above 52°C. Type I receptors were first identified by (Burgess & Perl, 1967) and are referred to as high-threshold mechanoreceptors. Type II Aδ-nociceptors are sensitive to mechanical stimuli and noxious heat, but their temperature threshold is near 43°C, similar to that of C-fibres. Type II Aδ-fibres also have a lower mean conduction velocity of 15 m/s and respond to bradykinin, prostaglandins and protons (Lang *et al.*, 1990; Martin *et al.*, 1987; Steen *et al.*, 1992).

Anatomy of the Dorsal Horn

The dorsal horn of the spinal cord is involved in the processing of sensory information. The detailed anatomy of the dorsal horn was first described by (Rexed, 1952). In the cat, it is subdivided into six parallel layers based upon the size of the neuronal cell bodies, their morphological features and their packing density. A similar organization of the dorsal horn has been described in the rat (Molander *et al.*, 1984). The two most superficial layers of the dorsal horn, lamina I and lamina II, are commonly referred to as the marginal zone and the substantia gelatinosa, respectively. These regions receive Aδ- and C-fibre input and are important for processing nociceptive information (Lamotte *et al.*, 1976; Light & Perl, 1979a; Snider & McMahon, 1998; Terman & Bonica, 2001). Innervation of lamina I and II by Aδ- and C-fibres is shown schematically in Figure 1-1.

Nociceptive information is transmitted from the spinal cord to the brain via several tracts (Brodal, 1992; Terman & Bonica, 2001). Among them the spinothalamic tract (STT) and the spinoreticular tract (SRT) are regarded as the most important pathways for conveying nociceptive information.

Spinothalamic Tract

Most of the axons in the STT originate in lamina I or V, cross to the other side of the spinal cord and ultimately terminate in the thalamus. Spinothalamic neurons respond to various stimuli and are classified by their response properties: (i) low-threshold neurons—cells that respond to light mechanical stimulation; (ii) wide-dynamic range neurons—cells that respond to both nociceptive and light mechanical stimulation; (iii) high-threshold neurons—cells that only respond to stimuli that are sufficient to activate nociceptors; and (iv) thermosensitive neurons—cells that respond to warming or cooling of the skin (Brodal, 1992).

Spinoreticular Tract

The SRT plays an important role in pain modulation. The SRT comprises a variety nuclei including the medullary, pontine and mesencephalic nuclei (Brodal, 1992; Terman & Bonica, 2001). SRT neurons originate primarily in lamina VII and VIII, but some are also present in lamina I, V and X. The SRT likely plays a role in arousal in response to pain and participates in descending pain modulation (Basbaum & Fields, 1984).

Lamina I

Lamina I neurons receive input from skin, muscle, teeth, cornea, airways, viscera, joints and dura (reviewed by Craig, 1996) and consequently project to a number of different regions in the CNS. Many spinal cord lamina I neurons send projections to sympathetic nuclei that may play a role in regulating vasoconstrictor activity (Craig & Hunsley, 1991; Janig, 1988). In the rat, there is a direct spino-hypothalamic projection originating in lamina I (Burnstein, 1996; Dado *et al.*, 1994). Lamina I neurons also project to a number of sites in the medulla, pons and mesencephalon. These include, but are not limited to: (1) the ventrolateral medulla—a region involved in cardiorespiratory function; (2) the solitary nucleus, which may modulate both visceral and baroreceptive responses; and (3) the periaqueductal grey (PAG)—a limbic structure that is involved in a variety of functions, including pain modulation. The lateral and ventrolateral PAG are responsible for descending modulation of dorsal horn neurons and receive input from lamina I. Lamina I neurons also project to thalamic nuclei involved in pain and temperature sensation and these nuclei project to the insular cortex. Therefore, the diverse projections of lamina I neurons provide a mechanism for regulating autonomic and neuroendocrine reflexes, as well as emotional and cognitive behaviours in response to pain.

Lamina I neurons can be subdivided into different categories based upon cell body shape and dendritic morphology (Gobel, 1978b; Lima & Coimbra, 1986; Zhang *et al.*, 1996a). Neurons in the cat trigeminal nucleus caudalis, a brainstem region analogous to the dorsal horn of the spinal cord, have been categorized into two types of pyramidal cells (spiny and smooth) and two types of multipolar cells (compact and loose). Spiny pyramidal cells typically have a dendritic arbour that extends out from the cell body by about 250 μm and their dendritic spines are numerous and closely spaced. By contrast, the dendrites of smooth pyramidal cells extend up to 700 μm from the cell body and have fewer, more widely spaced dendritic spines. Both spiny and smooth pyramidal cells have similar dendritic branching patterns. Compact multipolar cells have a compact dendritic arbour, typically about 50 μm wide, with most of the branches lying to one side of the cell body. Loose multipolar cells have a more dispersed dendritic arbour, about 200 μm wide. Common features of both types of multipolar cells are dendrites that extend from the cell body, turn sharply and run recurrently back to the cell body. Giant projection neurons (marginal cells of Waldeyer) are also present in lamina I, but these represent a small portion of the cell population (Chung *et al.*, 1984; Willis & Coggeshall, 1991). However, Waldeyer cells receive a very high density of γ-amino-butyric acid (GABA)-positive inputs to their cell bodies and proximal dendrites (Puskár *et al.*, 2001) suggesting they are under strong inhibitory control.

In rat spinal cord, four cell types have been identified in lamina I: (1) fusiform spiny neurons; (2) pyramidal neurons; (3) flattened aspiny neurons; and (4) multipolar neurons. With the exception of multipolar neurons, the dendrites of these cells remain confined to lamina I (Lima & Coimbra, 1986). Recently, the morphological and intrinsic membrane properties have been correlated for rat spinal cord lamina I neurons (Prescott & de Koninck, 2002). Cells that fire tonic action potentials (APs) are typically fusiform, whereas pyramidal cells exhibit phasic action potential firing patterns. Neurons that fire only single action potentials or have a delayed onset for spiking are multipolar neurons. The circuitry of lamina I is illustrated schematically in Figure 1-2.

Lamina II

Lamina II (substantia gelatinosa) neurons have been studied at length and two principle cell types have been identified. Stalked cells and islet cells have been identified in cat trigeminal nucleus caudalis and in rodent and cat lumbar spinal cord (Bennett *et al.*, 1980; Gobel, 1978a; Todd & Lewis, 1986) and correspond to the central and limiting cells described by Ramon y Cajal (Ramon y Cajal, 1909). Stalked cells are typically localized to the outer portions of lamina II and are identified by their small, round cell bodies and short, stalk-like dendrites. Their dendrites extend ventrally and receive input from lamina III glomeruli while their axons synapse with projection neurons in lamina I. Stalked cells are thought to be excitatory interneurons. Islet cells are often clustered together and their projections are confined almost exclusively to lamina II. Unlike those of stalked cells, islet cell dendrites extend rostrocaudally up to 500 μm and often show recurrent branching. Islet cells also produce nitric oxide, which may regulate the release of neuropeptides from primary afferents (Aimar *et al.*, 1998). It is likely that islet cells form the major population of inhibitory interneurons in lamina II. Additionally, two other kinds of inhibitory interneurons are present in cat trigeminal nucleus caudalis: the arboreal cell and the II-III border cell (Gobel, 1978a). The dendrites of the arboreal cell are localized predominantly to lamina II and extend a relatively short distance (~250 μm) rostrocaudally. The II-III border cell comprises a small portion of the cell population in lamina II. Its cell body is found on the border between lamina II and III or deep in lamina II and its dendrites extend ~400 μm in the rostrocaudal axis. The circuitry of lamina II is illustrated schematically in Figure 1-1.

Recently, Grudt & Perl (2002) completed a detailed analysis of the morphology, membrane properties and synaptic inputs for hamster spinal cord lamina I and II cells. They have subdivided lamina II neurons into five morphological categories: islet, central, medial—lateral, radial and vertical cells.

Islet cells are characterized by a dendritic tree which is markedly elongated in the rostrocaudal axis with limited spread in the dorsoventral and mediolateral axes. Islet cells have a resting potential at least 10 mV less negative than other cell types and display sustained AP discharge in response to depolarizing current. They also express H-currents (I_h) and modest inward-rectification in response to hyperpolarizing voltage commands. They generate characteristically large monosynaptic excitatory postsynaptic currents (EPSCs) upon dorsal root stimulation.

Central cells lie in the midline of the inner and outer parts of lamina II and have a moderately dense dendritic arbour which is oriented rostrocaudally and is much smaller than that found in islet cells. These cells are a functionally diverse group. Tonic central cells respond with repetitive discharge of APs in response to depolarizing current. Transient central cells respond with a few APs prior to falling silent in the presence of depolarizing current. Most transient central cells exhibit large GABAergic inhibitory postsynaptic currents (IPSCs). Some of these cells exhibit transient outward K^+ currents (A-currents, I_A), whereas others do not.

Medial—lateral cells have sparse dendritic trees that are oriented in the mediolateral and dorsoventral plane. These cells exhibit I_A, but, unlike transient central cells, exhibit tonic discharge in response to current injection and a high frequency of spontaneous EPSCs.

Radial (stellate) cells are characterized by a dendritic tree that radiates in all directions. These cells exhibit a characteristic delay in AP discharge following the injection of depolarizing current. The ensuing discharge tends to be irregular. These cells also exhibit inward-rectification and a weak I_H.

Vertical neurons likely correspond to the stalked cells defined by (Gobel, 1978a). These cells are oriented vertically with sparse, wide dendrites. Vertical cells never show transient AP discharge and always show tonic or delayed discharge in response to depolarizing current. They are further characterized by both fast (Aδ) monosynaptic EPSCs, as well as large, slow polysynaptic EPSCs following dorsal root stimulation. These cells also have a high frequency of spontaneous EPSCs.

Recent *in vivo* electrophysiological recordings from cat and rat spinal cord have correlated peripheral receptive fields with cell morphology (Furue *et al.*, 1999; Han *et al.*, 1998; Light & Willcockson, 1999). Fusiform and multipolar cells in lamina I were identified as nociceptive-specific, while islet, stalked and stellate (radial) cells responded to nociceptive and innocuous mechanical stimulation (Light & Willcockson, 1999). These are similar to results obtained *in vitro* (Grudt & Perl, 2002; Woolf & Fitzgerald, 1983).

Synaptic Transmission in the Dorsal Horn

The primary afferent inputs to lamina I and lamina II have been well characterized. These laminae receive inputs from Aδ and C-fibre nociceptors, as well as Aδ and C-fibre receptors that respond to innocuous thermal and mechanical stimuli (Light *et al.*, 1979; Light & Perl, 1979b; Sugiura *et al.*, 1986; Sugiura *et al.*, 1993). The termination of Aδ- and C-fibre inputs to laminae I and II are illustrated schematically in Figure 1-1.

In rat lamina II both fast excitatory postsynaptic potentials (fast EPSPs) and slow EPSPs arising from primary afferent fibres have been described (Yoshimura & Jessell, 1989). Both Aδ- and C-fibres are responsible for the fast EPSP, whereas the slow EPSP is mediated by Aδ fibres (Yoshimura & Jessell, 1989). The fast excitatory synaptic response is caused by the release of glutamate from the primary afferents (Schneider & Perl, 1988; Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1993) which activates postsynaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors (Bardoni *et al.*, 1998; Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1992; Yoshimura & Nishi, 1993). By contrast the slow EPSP can be is sensitive to NMDA receptor and tachykinin antagonists (Yoshimura, 1996). These findings have been extended with *in vivo* patch clamp recordings and demonstrate that EPSCs can be evoked by noxious and innocuous stimuli (Furue *et al.*, 1999). Recently, kainate receptors have been shown to play an important role in modulating nociceptive sensory transmission (Kerchner *et al.*, 2001; Kerchner *et al.*, 2002; Li *et al.*, 1999). A novel feature of these receptors is their ability to regulate GABA and glycine release by a presynaptic ionotropic mechanism in dorsal horn neurons (Kerchner *et al.*, 2001).

In lamina I, projection neurons are glutamate-immunoreactive and colocalize with the Ca^{2+}-binding protein, calbindin-D28K, which is thought to be a marker for excitatory neurons, thus suggesting these cells are excitatory neurons (Gamboa-Esteves *et al.*, 2001a; Gamboa-Esteves *et al.*, 2001b). In lamina II, immunocytochemical identification of excitatory neurons is ambiguous (Todd & Spike, 1993). On the basis of morphology and cell location, Gobel has proposed that stalked cells are likely excitatory interneurons (Gobel, 1978a). Similar to lamina I neurons, putatative excitatory interneurons in lamina II are immunoreactive for the calcium binding protein, calbindin-D28K (Antal *et al.*, 1991; Polgar & Antal, 1995). Neurons that express high levels of the GluR2/3 subunits, which confers Ca^{2+}-permeability to the AMPA receptor, do not contain GABA, suggesting they may be excitatory interneurons (Kerr *et al.*, 1998).

GABA and glycine are the predominant inhibitory neurotransmitters in lamina I and II (reviewed by (Malcangio & Bowery, 1996; Todd & Spike, 1993)). GABA immunoreactive (GABA-IR) neurons are present in lamina II and comprise ~30% of the total population of neurons in this region (Magoul *et al.*, 1987; Todd & McKenzie, 1989; Todd & Sullivan, 1990). These GABA-IR cells have been identified as two populations of islet cells: large islet cells which are GABA-IR and small islet cells which are not GABA-IR (Spike & Todd, 1992; Todd & McKenzie, 1989). GABA immunoreactivity is detected in lamina II synapses and is thought to originate from local inhibitory interneurons (Todd, 1996). $GABA_A$ receptor binding sites are present on fine diameter primary afferent fibres that are sensitive to capsaicin (Coggeshall & Carlton, 1997; Singer & Placheta, 1980).

Stimulation of Aδ-fibres results in fast EPSCs that are often followed by a short or long duration inhibitory postsynaptic potential (IPSP) or IPSC (Yoshimura & Nishi, 1993; Yoshimura & Nishi, 1995). These IPSPs/IPSCs are sensitive to the $GABA_A$ receptor antagonist, bicuculline, and the glycine receptor antagonist, strychnine. These IPSPs/IPSCs have variable latencies and exhibit failures following high frequency stimulation, suggesting they are polysynaptic in origin (Yoshimura & Nishi, 1993;

Yoshimura & Nishi, 1995). Similarly, in trigeminal nucleus pars caudalis evoked IPSCs and TTX-insensitive miniature IPSCs are sensitive to bicuculline and strychnine (Grudt & Henderson, 1998). *In vivo* patch-clamp recordings have suggested that GABAergic and glycinergic IPSCs are activated by innocuous mechanical stimulation (Narikawa *et al.*, 2000).

Glycine immunoreactivity (glycine-IR) has been detected in lamina I and II and is typically colocalized in cell bodies that are GABA-IR (Todd, 1990; Todd, 1996; Todd & Sullivan, 1990). Cells which are immunoreactive for both GABA and glycine are indistinguishable from neurons that are only GABA-IR (Spike & Todd, 1992; Todd & Sullivan, 1990) and GABA, glycine and their receptors are often colocalized at synapses in the dorsal horn (Todd *et al.*, 1996). Furthermore, GABA and glycine are co-released from individual vesicles at some synapses in the dorsal horn (Chery & de Koninck, 1999) and ventral horn (Jonas *et al.*, 1998; O'Brien & Berger, 1999). Similarly, adenosine triphosphate (ATP) and GABA are co-released from the same vesicle in dorsal horn neurons (Hugel & Schlichter, 2000; Jo & Schlichter, 1999). ATP is then metabolized to adenosine and can finely tune GABAergic synaptic transmission (Jo & Schlichter, 1999).

In summary, glutamate is the predominant excitatory neurotransmitter in lamina I and II and it is released from Aδ and C primary afferent fibres. Although direct evidence is lacking, glutamatergic interneurons in lamina II are thought to be stalked cells or small islet cells. GABA and glycine are responsible for mediating inhibitory transmission. These neurotransmitters are often co-released from the same vesicle and are likely released from islet cells. Finally, *in vivo* patch-clamp recordings have shown that EPSCs and IPSCs are evoked by different peripheral stimuli.

Pathological Modification of Pain Pathways

Neuropathic Pain

The aforementioned description of the anatomical and functional organization of the dorsal horn is pertinent to normal nociceptive pain transmission. Nociceptive pain is described as "good" pain because it serves a protective function following injury (Iadarola & Caudle, 2002). By contrast, neuropathic pain which is initiated following a lesion to the nervous system and is caused by pathological changes to a nerve (mononeuropathy) or nerves (polyneuropathy) (Merskey, 1986) represents "bad" pain, as it serves no protective function (Iadarola & Caudle, 2002) and characteristically persists long after the original injury has resolved. Moreover, neuropathic pain is generally insensitive to opioid analgesics. Therefore, one aspect of this thesis is to examine potential drug targets for the treatment of neuropathic pain.

The etiology of neuropathic pain is diverse, however, there are certain clinical features common to neuropathic pain (Scadding, 1999). These are summarized in Table 1-2 (modified from Scadding, 1999).

Animal Models of Neuropathic Pain

Animal models have been developed which mimic different clinical aspects of neuropathic pain. A common feature of these neuropathic pain models is partial or complete denervation.

Neuroma Model

The first animal model of peripheral neuropathy was developed by Wall and colleagues (Wall *et al.*, 1979b; Wall *et al.*, 1979a; Wall & Gutnick, 1974). The neuroma model is produced by completely transecting the sciatic nerve. The nerve is cut and resutured or sealed in a polyethylene tube. This model is thought to replicate human syndromes observed following limb amputation or stroke (Bowsher, 2002) and is often accompanied by autotomy, the self-mutilation of the denervated limb (Blumenkopf & Lipman, 1991; Coderre *et al.*, 1986; Coderre & Melzack, 1986; Devor, 1991; Wall *et al.*, 1979a). A possible mechanism for autotomy may be due to in part to the animal's response to spontaneous pain as consequence of injury discharge from C-fibres that modifies the excitability of dorsal horn neurons (Seltzer *et al.*, 1991). However, the exact relationship between neuropathic pain and autotomy continues to be the subject of debate (Kauppila, 1998; Kruger, 1992; Rodin & Kruger, 1984).

Chronic Constriction Model

The chronic constriction injury model (CCI) is produced by loose ligation of the sciatic nerve (Bennett & Xie, 1988). The ligation selectively axotomizes large diameter myelinated fibres, while the remaining small diameter fibres are left intact (Kajander & Bennett, 1992). The CCI also produces abnormal pain sensations including spontaneous pain, allodynia and hyperalgesia (Bennett, 1993; Bennett & Xie, 1988). Many of these symptoms are reduced or abolished following sympathectomy, suggesting an interaction between the autonomic and sensory nervous systems (Bennett, 1993; Wakisaka *et al.*, 1991a). The CCI model has been refined by loosely enclosing the sciatic nerve with a polyethylene cuff of fixed diameter (Mosconi & Kruger, 1996). This model produces pain-related behaviours and axonal pathology similar to the CCI.

Spinal Nerve Ligation Model

The spinal nerve ligation model (SNL) of neuropathic pain is produced by a tight ligation of the lumber 5 and 6 spinal nerves. It produces mechanical allodynia, hyperalgesia and spontaneous pain (Kim & Chung, 1992). However, the SNL model produces greater allodynia when compared to the CCI (Kim *et al.*, 1997a).

Thus, the neuroma, CCI and SNL models all produce symptoms of neuropathic pain and they may be representative of different populations of neuropathic pain patients.

Anatomical and Pathophysiological Substrates for Neuropathic Pain

Following peripheral nerve damage, primary afferent fibres often become spontaneously active (Devor & Seltzer, 1999; Tal & Eliav, 1996; Wall & Gutnick, 1974). This ectopic activity depends on accumulation of voltage-sensitive Na^+ channels at the site of nerve injury (Devor *et al.*, 1993; Matzner & Devor, 1994; Omana-Zapata *et al.*, 1997b; Omana-Zapata *et al.*, 1997a). Spontaneous activity also develops in the dorsal root ganglia (DRG) following neuroma formation or after a CCI (Kajander *et al.*, 1992; Wall & Devor, 1983). This ectopic discharge may be enhanced by the sprouting of noradrenergic sympathetic fibres into the DRG, where they form basket-like structures around large diameter DRG neurons (Lee *et al.*, 1998; McLachlan *et al.*, 1993; Ramer & Bisby, 1997). These unusual connections are of interest because nerve-injured DRG begin to express α-adrenoceptors (Abdulla & Smith, 1997a; Devor *et al.*, 1994; Sato &

Perl, 1991) and many neuropathic pain symptoms are reduced following sympathectomy (Kim *et al.*, 1997a).

Peripheral nerve injury is also thought to trigger a structural reorganization of myelinated primary afferent fibres in the dorsal horn (Koerber *et al.*, 1994; Shortland & Woolf, 1993; Woolf *et al.*, 1992). However, this structural reorganization has been recently questioned (Bao *et al.*, 2002; Tong *et al.*, 1999). After axotomy, large diameter Aβ-fibres, which normally terminate in the deeper laminae of the dorsal horn, form inappropriate synaptic connections in lamina II, which normally receives input from Aδ- and C-fibre nociceptors. This sprouting is a long-lasting, but not a permanent, characteristic of peripheral axotomy and is due, in part, to atrophic loss of central terminals (Woolf *et al.*, 1995). The anatomical reorganization is accompanied by functional changes in synaptic inputs to lamina II (Kohama *et al.*, 2000; Okamoto *et al.*, 2001). After axotomy there are fewer slow-conducting, high-threshold inputs into lamina II and there is an increase in the number of low-threshold, fast-conducting inputs, characteristic of Aβ-fibres, into lamina II (Kohama *et al.*, 2000). Peripheral axotomy also produces an increase in the number of Aβ-fibre-mediated EPSCs in lamina II (Okamoto *et al.*, 2001). Since most of these responses are polysynaptic it is likely that a large proportion of these fibres form synaptic connections with interneurons in lamina II (Okamoto *et al.*, 2001).

Peripheral nerve injury also causes lamina II neurons to become hyperexcitable (Colvin *et al.*, 1996; Dalal *et al.*, 1999; Laird & Bennett, 1993; Omana-Zapata *et al.*, 1997b; Omana-Zapata *et al.*, 1997a). Axotomy or a CCI cause neurons in the superficial dorsal horn to show abnormal characteristics, such as a response to gentle mechanical stimulation near the nerve injury site, lack of peripheral receptive fields and spontaneous activity (Dalal *et al.*, 1999; Laird & Bennett, 1993). Most cells with intact peripheral receptive fields, which respond to gentle mechanical stimulation, are also activated by C-fibre input (Laird & Bennett, 1993). Partial nerve injury appears to cause a selective loss of GABAergic inhibition in the dorsal horn which may result in an amplification of excitatory synaptic input into this region (Moore *et al.*, 2002). Other mechanisms that contribute to dorsal horn neuron hyperexcitability may result from changes in NMDA receptor or Ca^{2+} channel function (Isaev *et al.*, 2000; Kawamata & Omote, 1996; Kim *et al.*, 1997b; Parsons, 2001). Second messengers may also play a role because mice lacking protein kinase C gamma (PKCγ) do not develop neuropathic pain (Malmberg *et al.*, 1997). Additionally, computer modeling studies of dorsal horn neurons demonstrate that changes in Na^+ channel distribution may contribute to spontaneous activity in these cells (Safronov *et al.*, 2000).

An important and interesting feature of neuropathic pain is the limited efficacy of opioids for its management (Arner & Meyerson, 1988; Dickenson, 1994). Several lines of evidence support the lowered clinical efficacy of the preferential μ-agonist, morphine, in the treatment of neuropathic pain. In animal models of neuropathic pain, morphine has a relatively weak spinal analgesic action (Nichols *et al.*, 1995; Ossipov *et al.*, 1995; Yamamoto *et al.*, 1994) and immunoreactivity for μ- and δ-opioid receptors is reduced in the DRG and dorsal horn of the spinal cord following peripheral nerve axotomy (de Groot *et al.*, 1999; Zhang *et al.*, 1998a). The effect of morphine on N-type Ca^{2+} currents in DRG cell bodies is attenuated in nerve-injured (axotomized) animals (Abdulla &

Smith, 1998). Thus, non-opioid, *spinal peptidergic* mechanisms represent a potential target for the treatment of neuropathic pain.

Neuropeptides in Pain Pathways

Various neuropeptides are expressed in DRG and dorsal horn neurons (Coggeshall & Carlton, 1997; Jessell & Dodd, 1989; Todd & Spike, 1993). Because expression of many of these neuropeptides is dramatically altered following peripheral nerve injury, it may be suggested that they play a role in the development of neuropathic pain (Dray, 1996; Hokfelt *et al.*, 1994). Some of the best-studied neuropeptides include Substance P, CGRP, galanin, NPY, endogenous opioids and nociceptin (N/OFQ). Substance P and CGRP have excitatory actions and are generally pro-nociceptive, whereas NPY, opioids and N/OFQ are generally inhibitory and are anti-nociceptive.

Substance P

Substance P is an 11-amino acid peptide that is important for pain signaling and is one of the best-studied members of the tachykinin family (Helke *et al.*, 1990; Ribeiro-da-Silva & Hokfelt, 2000). Substance P is the preferential agonist for the neurokinin-1 (NK-1) receptor which is a member of the G-protein-coupled receptor (GPCR) superfamily (Maggi & Schwartz, 1997; Regoli *et al.*, 1994). Substance P is present in the DRG and dorsal horn (Hokfelt *et al.*, 1975) and is localized to primary afferent fibres (Jessell *et al.*, 1979). Substance P-immunoreactive (Substance P-IR) fibres are present throughout lamina I and the outer portions of lamina II (Hokfelt *et al.*, 1975; Ribeiro-da-Silva *et al.*, 1989). Immunoreactivity is substantially diminished in the ventral part of lamina II and III and most Substance P-immunoreactivity is represented by fibres crossing towards deeper laminae (Ruda *et al.*, 1986). An interesting characteristic of Substance P-immunoreactivity in dorsal horn neurons is that it is usually colocalized with enkephalin (Ribeiro-da-Silva *et al.*, 1991).

NK-1 receptors are present in laminae I and II. In lamina I, most fusiform cells and some larger marginal cells of Waldeyer, with dendrites that extend into laminae II and III, are NK-1-positive (Littlewood *et al.*, 1995). Labeled cells in lamina II are sparse and are weakly labeled. Neither stalked nor islet cells label for NK-1, which is of interest because stalked cells are thought to relay information between small diameter primary afferent fibres and projection neurons in lamina I (Brown *et al.*, 1995; Littlewood *et al.*, 1995). Recently, μ-opioid receptors were shown to frequently colocalize with NK-1 receptors on unmyelinated primary afferents in the trigeminal dorsal horn (Aicher *et al.*, 2000). Because opioids decrease Substance P release in the dorsal horn (Jessell & Iversen, 1977; Yaksh *et al.*, 1980) this may contribute to opioid analgesia at the spinal level (Trafton *et al.*, 1999).

Substance P is released *in vivo* in response to noxious stimulation from nociceptive primary afferents that terminate in the superficial dorsal horn (Allen *et al.*, 1997; Duggan *et al.*, 1987; Duggan *et al.*, 1988b). These Substance P-containing afferent fibres preferentially terminate on nociceptive-specific neurons in the dorsal horn (de Koninck *et al.*, 1992; Ma *et al.*, 1996). Substance P is also responsible for eliciting a slow EPSP/EPSC in mammalian dorsal horn neurons (de Koninck & Henry, 1991; Li & Zhuo, 2001; Murase *et al.*, 1982; Yoshimura *et al.*, 1993) and lamprey dorsal horn neurons (Parker & Grillner, 1996). This slow EPSP may be mediated by the activation of a persistent, slow, inward Ca^{2+} current (Murase *et al.*, 1986; Murase *et al.*, 1989).

NK-1 receptor activation by Substance P is critical for the development of central sensitization of dorsal horn neurons (Dougherty *et al.*, 1994; Ma & Woolf, 1995; Xu *et al.*, 1992) and modulation of wind-up (Baranauskas & Nistri, 1998; Budai & Larson, 1996; Kellstein *et al.*, 1990). Recently, the NK-1 receptor has proven to be important for the induction of long-term potentiation (LTP) between primary afferent C-fibres and second-order neurons in the dorsal horn (Liu & Sandkuhler, 1998; Sequeira & Nässtrom, 1998). This may be an important cellular mechanism underlying hyperalgesia in inflammatory and neuropathic pain (Woolf & Salter, 2000; Zimmermann, 2001). However, Substance P antagonists are generally ineffective for the management of inflammatory and neuropathic pain in humans (Goldstein *et al.*, 2000; Goldstein *et al.*, 2001).

Calcitonin Gene-Related Peptide

Calcitonin Gene-Related Peptide (CGRP) is a 37-amino acid peptide and two main forms have been identified: α-CGRP and β-CGRP (Merighi, 2002). α-CGRP is the predominant form present in pain pathways (Merighi, 2002). CGRP immunoreactivity is present in small diameter, nociceptive-specific DRG neurons (Ishida-Yamamoto & Senba, 1990; Lawson *et al.*, 1996; McCarthy & Lawson, 1990; Villar *et al.*, 1989) and in the central terminals of the dorsal horn (Henry *et al.*, 1993; Kar & Quirion, 1995; Todd, 1997; Yashpal *et al.*, 1992). The majority of CGRP immunoreactivity disappears after dorsal root section, suggesting it is confined to primary afferent fibres (Chung *et al.*, 1988; Traub *et al.*, 1989a; Traub *et al.*, 1989b).

Although, CGRP receptor labeling in laminae I and II is sparse (Kar & Quirion, 1995; Todd, 1997) and cell bodies in these laminae are generally believed not to contain CGRP, it has similar actions to Substance P in the spinal cord. CGRP is released in the dorsal horn in response to noxious heat or to locally applied chemical irritants (Garry *et al.*, 2000; Garry & Hargreaves, 1992; Morton & Hutchison, 1989). Like Substance P, CGRP causes a slow depolarization or slow EPSP (Miletic & Tan, 1988; Ryu *et al.*, 1988; Woodley & Kendig, 1991) and facilitates glutamatergic responses in dorsal horn neurons (Ebersberger *et al.*, 2000; Leem *et al.*, 2001). Intrathecal application of CGRP also causes hyperalgesia (Coderre & Melzack, 1991). Interestingly, CGRP and Substance P are often colocalized to the same vesicle (Merighi, 2002) and electrophysiological (Biella *et al.*, 1991) and behavioural experiments have suggested that these peptides act synergistically in animal pain models (Wiesenfeld-Hallin *et al.*, 1984; Woolf & Wiesenfeld-Hallin, 1986).

Galanin

Galanin is a 29-amino acid peptide originally isolated from porcine intestine (Tatemoto *et al.*, 1983). To date, three galanin receptors, GalR1, GalR2 and GalR3 have been characterized and cloned and all are members of the GPCR superfamily (Branchek *et al.*, 2000).

Galanin may play an important role in neuropathic pain. Under normal conditions it is virtually absent from the DRG, but following sciatic nerve transection or crush its levels increase dramatically in neurons of all sizes in the DRG (Ikeda *et al.*, 1997; Villar *et al.*, 1989). Galanin and its receptors are also present in the DRG and superficial dorsal horn (Kar & Quirion, 1994; Kar & Quirion, 1995; Melander *et al.*, 1986a; Melander *et al.*, 1986b; Zhang *et al.*, 1995a). Their expression in the DRG is differentially affected by axotomy: GalR1-mRNA is downregulated, while only small decreases in GalR2-mRNA are observed (Zhang *et al.*, 1998b). Galanin-immunoreactivity (Gal-IR) is present on many primary afferent terminals innervating lamina II which also contain Substance P and CGRP (Zhang *et al.*, 1993). Within lamina II Gal-IR is localized primarily to islet cells and is mainly colocalized with enkephalins, but sometimes also with neuropeptide Y (Zhang *et al.*, 1995b). These findings have been extended by Todd and colleagues (Simmons *et al.*, 1995) who show galanin is contained only in local GABAergic interneurons in laminae I-III.

Galanin has complex actions at the spinal level. It is released from C-fibres in the dorsal horn of neuropathic rats (Colvin *et al.*, 1997; Colvin & Duggan, 1998) and attenuates the release of norepinephrine (Reimann & Schneider, 1993). In intact rats, galanin enhances Aδ- and C-fibre-evoked responses, post-discharge and wind-up (Reeve *et al.*, 2000). In rats with sciatic nerve ligation it dose-dependently suppresses the activity of dorsal horn neurons (Wagner *et al.*, 1998). Furthermore, intrathecal injection of galanin depresses C-fibre-evoked flexor reflexes (Wiesenfeld-Hallin *et al.*, 1989). However, this action is biphasic, as low doses consistently facilitate flexor reflexes, while high doses suppress them (Holets *et al.*, 1988; Xu *et al.*, 1990; Yashpal *et al.*, 1992). In addition, galanin decreases the nociceptive threshold to mechanical, but not thermal, stimuli (Gobel, 1978a; Kuraishi *et al.*, 1991). These paradoxical actions of galanin may be due to receptor specific actions: low-dose galanin activates GalR2 and has a pro-nociceptive action, whereas high-dose galanin acts via GalR1 to reduce allodynia following a CCI (Bennett *et al.*, 1980).

Endogenous Opioids

Opioids, such as morphine, and endogenous opioid peptides (Hughes *et al.*, 1975; Lord *et al.*, 1977) act at receptors in the peripheral and central nervous systems to produce analgesia (Pasternak, 1993; Yaksh, 1997). Endogenous opioid peptides are derived from three large precursors: pro-opiomelanocortin, the precursor for β-endorphin; proenkephalin A, the precursor for Leu- and Met-enkephalins; and prodynorphin, the precursor for dynorphin peptides (Loh *et al.*, 1984). Opioids and endogenous opioid peptides exert their effects by interacting with one or more subclasses of three opiate receptors: μ, δ, and κ (Knapp *et al.*, 1995; Pasternak, 1993; Satoh & Minami, 1995; Yaksh & Noueihed, 1985). Recent IUPHAR guidelines (Dhawan *et al.*, 1996) have recommended that the members of the opioid receptor family should be designated as OP (opioid peptide) with numeric subscripts indicating the chronological cloning of the receptors, thus OP1, OP2 and OP3 correspond to the δ-, κ-, and μ-opioid receptors, respectively. These receptors are members of the GPCR superfamily and are prototypical G_i/G_o-coupled receptors. All three receptors inhibit cyclic AMP (cAMP) formation (Knapp *et al.*, 1995; Law *et al.*, 2000; Satoh & Minami, 1995).

μ, δ and κ-opioid receptors have affinity for the opiate analgesic morphine, with the μ-receptor exhibiting approximately 100 times greater affinity for morphine than the δ- and κ-receptors (Martin, 1983; Pasternak, 1993). In addition, opioid receptors are activated by endogenous ligands. Enkephalins appear to be the endogenous ligands at μ-

and δ-receptors (Glazer & Basbaum, 1981; Hunt *et al.*, 1980), whereas dynorphin A_{1-17} is likely the endogenous ligand at the κ-receptor (Chavkin *et al.*, 1982). Endomorphin-1 and endomorphin-2 are likely the endogenous ligands for the μ-opioid receptor (Horvath, 2000; Zadina *et al.*, 1997). These peptides exhibit greater affinity for the μ-receptor than the μ-agonist, DAMGO and produce prolonged analgesia in mice (Zadina *et al.*, 1997).

Opioid receptors and opioid peptides are distributed widely in regions of the CNS and PNS that are involved in modulating pain behaviour (Yaksh, 1997). However, particularly intense expression of opioid receptor mRNA and opioid receptor-immunoreactivity are present in DRG neurons (Ji *et al.*, 1995; Maekawa *et al.*, 1994; Zhang *et al.*, 1998a) and superficial portions of the dorsal horn (laminae I and II) of the spinal cord (Besse *et al.*, 1990; Morris & Herz, 1987; Stevens *et al.*, 1991). These observations suggest that primary afferent neurons and their terminals in the dorsal horn are important targets for the analgesic effects of opioids.

Analgesic Actions of Opioids: Presynaptic Mechanisms

Analgesic actions of opioids within the dorsal horn involve presynaptic mechanisms to reduce neurotransmitter release from primary afferent terminals in laminae I and II (Jessell & Iversen, 1977; Yaksh *et al.*, 1980). A presynaptic site of action of opioids in the dorsal horn is supported by ligand binding and electrophysiological studies. Binding sites for μ-, δ- and κ-opioids are present in laminae I and II of the dorsal horn (Gouarderes *et al.*, 1985; Morris & Herz, 1987). Furthermore, dorsal rhizotomy decreases the density of μ-, δ- and κ-binding sites in the dorsal horn, indicating that at least a proportion of the these receptors are located on primary afferent terminals (Besse *et al.*, 1990; Lamotte *et al.*, 1976).

Several groups have shown that μ-, δ- and κ-opioids reduce the amplitude of EPSPs and EPSCs (Hori *et al.*, 1992; Randic *et al.*, 1995). μ-opioids also suppress IPSCs in substantia gelatinosa neurons (Grudt & Henderson, 1998; Kerchner & Zhuo, 2002; Moran & Smith, 2002) but this finding is controversial (Kohno *et al.*, 1999). Support for a presynaptic site of action is provided by two observations: (1) opioids reduce the frequency of miniature EPSCs (mEPSCs) (Hori *et al.*, 1992; Kohno *et al.*, 1999) and miniature IPSCs (mIPSCs) (Grudt & Henderson, 1998) without altering their amplitude distribution; and (2) opioids reduce the amplitude of evoked-EPSCs without affecting post-synaptic responses to exogenously applied glutamate or glutamate agonists (Glaum *et al.*, 1994; Hori *et al.*, 1992). Furthermore, (Hori *et al.*, 1992) suggest suppression of Ca^{2+} current at the primary afferent terminals in lamina I contributes to the analgesic effects of opioids. This observation is consistent with the activation of opioid receptors on the cell bodies of DRG neurons which leads to suppression of high-voltage activated Ca^{2+} current (HVA I_{Ca}) or Ca^{2+}-dependent APs (Abdulla & Smith, 1998; Moises *et al.*, 1994; Rusin & Moises, 1995; Schroeder *et al.*, 1991; Taddese *et al.*, 1995; Werz & Macdonald, 1982; Womack & McCleskey, 1995). These actions of opioids on the DRG cell bodies may, therefore, reflect their action at the primary afferent terminals in the spinal cord.

To summarize, opioid receptor activation produces analgesia, which appears to be mediated to some extent at the level of the spinal cord. Binding studies indicate opioid receptors are present on primary afferent fibres which terminate in the substantia gelatinosa. Activation of these presynaptic opioid receptors inhibits transmitter release in the dorsal horn, thereby altering the inflow of nociceptive information. Lastly, this action may involve a reduction in Ca^{2+} influx at the primary afferent terminals.

Analgesic Actions of Opioids: Postsynaptic Mechanisms

In the dorsal horn, opioids also act postsynaptically, and this may contribute to their analgesic actions. *In vivo* morphine application inhibits AP discharge from spinal cord dorsal horn neurons (Duggan *et al.*, 1977; Johnson & Duggan, 1981; Jones *et al.*, 1990; Zieglgansberger & Bayer, 1976). μ-opioids also activate an inwardly-rectifying potassium conductance (GIRK) in substantia gelatinosa neurons (Grudt & Williams, 1994; Schneider *et al.*, 1998) which causes membrane hyperpolarization (Murase *et al.*, 1982; Yoshimura & North, 1983). However, these postsynaptic actions may not play a significant role in opioid-induced analgesia (Trafton *et al.*, 2000). μ-opioids can also modulate excitatory and inhibitory amino acid-evoked currents in dorsal horn neurons. μ-receptor activation reduces the amplitude of NMDA-evoked currents by acting directly at a site on the NMDA receptor (Rusin & Randic, 1991) or potentiates them by activating a PKC-dependent pathway (Chen & Huang, 1991). μ-opioids also reduce AMPA-activated currents in acutely isolated dorsal horn neurons via G-protein activation (Kolaj & Randic, 1996) and facilitate $GABA_A$-evoked currents in acutely isolated dorsal horn neurons (Wang & Randic, 1994).

The effects of κ- or δ-receptor activation appear to be similar to those observed following μ-receptor activation. κ-receptor activation reduces EPSP amplitude via pre- and postsynaptic mechanisms in substantia gelatinosa neurons (Randic *et al.*, 1995) and these receptors are likely coupled to an inwardly-rectifying K^+ conductance in these neurons (Grudt & Williams, 1993). Similarly, δ-receptor activation produces an outward current in a small number of substantia gelatinosa neurons (Glaum *et al.*, 1994).

In summary, opioid receptor activation exerts several postsynaptic effects on dorsal horn neurons. These include the suppression of AP discharge, activation of an inwardly-rectifying K^+ conductance which causes membrane hyperpolarization, and modulation of excitatory and inhibitory amino acid-mediated currents. The cellular effects of opioids are shown schematically in Figure 1-3.

The Role of Opioids in Descending Pain Modulation

Opioids also produce analgesia by their actions in the periaqueductal grey (PAG) (Behbehani, 1995; Christie *et al.*, 2000; Reichling *et al.*, 1988; Vaughan & Christie, 1997) and rostral ventromedial medulla (RVM) (Bennett *et al.*, 1980; Fields *et al.*, 1983; Ikeda *et al.*, 1997; Marinelli *et al.*, 2002). Within the PAG, μ-opioid receptor-mediated analgesia is caused by presynaptic inhibition of GABA release (Christie *et al.*, 2000; Osborne *et al.*, 1996; Vaughan & Christie, 1997). This, in turn, disinhibits PAG output projection neurons to regions such as the RVM (Osborne *et al.*, 1996) and to nociceptive neurons in the doral horn (Dickenson & Le Bars, 1983).

The role of opioids in the RVM is comparable to their actions in the PAG. In the RVM, two cell types are thought to be involved in pain modulation and have different responses to opioids (Fields *et al.*, 1991). On-cells discharge prior to the occurrence of a nociceptive reflex, contribute to pro-nociceptive processes and these cells are directly inhibited by opioids. Off-cells terminate firing prior to the occurrence of a nociceptive reflex and these cells are activated, via disinhibition, by opioids and mediate the analgesic effect of opioids. Furthermore, these off-cells are likely innervated by projection neurons from the PAG. RVM axon terminals form axosomatic and axodendritic synapses with local interneurons and projection neurons in the dorsal horn (Fields *et al.*, 1991) and may inhibit excitatory interneurons or activate inhibitory interneurons in lamina II, thus modulating nociception (Fields *et al.*, 1991). These effects of opioids in the PAG and RVM are described in greater detail below when comparing their actions with nociceptin/orphanin FQ.

Neuropeptide Y

Neuropeptide Y is a 36-amino acid polypeptide. It is one of the most widely distributed peptides in the mammalian central and peripheral nervous systems. NPY has a role in various physiological and pathophysiological conditions including autonomic regulation, circadian rhythms, epilepsy, food-intake and pain modulation (Colmers & Bleakman, 1994).

NPY Receptors

Five distinct NPY receptors have been cloned and characterized: Y1, Y2, Y4, Y5 and Y6. These receptors all belong to the heptahelical GPCR superfamily (Cabrele & Beck-Sickinger, 2000; Michel *et al.*, 1998). Sequence comparisons indicate that Y1, Y4, and Y6 receptors are more closely related to each other than Y2 and Y5 receptors (Larhammar, 1996). All NPY receptors are presumably G_i/G_o-coupled receptors, a conclusion based on their ability to inhibit adenylyl cyclase (Michel, 1991). These data are summarized in Table 1-3.

The Role of NPY in Primary Sensory Neurons and the Spinal Cord Dorsal Horn

NPY inhibits depolarization-induced Substance P release from DRG neurons (Walker *et al.*, 1988) and acetylcholine release from nodose ganglion neurons (Wiley *et al.*, 1990). NPY also inhibits action-potential induced increases in intracellular Ca^{2+} in DRG neurons (Bleakman *et al.*, 1991). In DRG neurons, these actions are thought to arise primarily by NPY's ability to suppress voltage-dependent calcium channels (VDCC) (Thayer & Miller, 1990; Walker *et al.*, 1988). This action of NPY on VDCCs is via a G-protein coupled mechanism (Ewald *et al.*, 1989). Activation of the Y2 receptor in DRG and nodose ganglion neurons suppresses ω-conotoxin-sensitive N-type Ca^{2+} current (Abdulla & Smith, 1999; Bleakman *et al.*, 1991; Wiley *et al.*, 1993), whereas, activation of Y1 receptors potentiates L-type Ca^{2+} current in these cells (Abdulla & Smith, 1999; Wiley *et al.*, 1993). Additionally, the action of NPY on N-type current is most pronounced in small DRG neurons (Abdulla & Smith, 1999) which are thought to be nociceptors (Bessou & Perl, 1969). Interestingly, in sympathetic neurons NPY has been shown to suppress N-type Ca^{2+} channels at their terminals (Toth *et al.*, 1993). If a similar mechanism is present in sensory neurons, it would suggest NPY plays a role in modulating nociceptive sensory information.

The modulation of nociceptive information by NPY is intriguing. NPY is virtually absent from DRG neurons in normal rats and is expressed only following peripheral nerve injury (Benoliel *et al.*, 2001; Noguchi *et al.*, 1993; Wakisaka *et al.*, 1991b). Therefore, alterations in NPY expression may be associated with the etiology of neuropathic pain. Thus, NPY in the somatosensory system may be a potential therapeutic target for neuropathic pain. NPY up-regulation occurs preferentially in large diameter

DRG neurons and concomitant increases are observed in deeper laminae of the spinal cord, areas to which large diameter primary afferent fibres project (Wakisaka *et al.*, 1991b; Wakisaka *et al.*, 1992). Although DRG neurons do not normally express NPY, receptors are present in both normal and nerve-injured animals. *In situ* hybridization studies indicate approximately 20% of small DRG neurons express Y1 receptors (Zhang *et al.*, 1994a) and about 15% of all DRG neurons express Y2 receptors (Zhang *et al.*, 1997). However, after axotomy Y1 receptor mRNA levels decrease (Zhang *et al.*, 1994b) whereas Y2 receptor mRNA levels increase, primarily in large diameter DRG neurons (Zhang *et al.*, 1997). These findings are consistent with binding studies of NPY in the DRG (Walker *et al.*, 1988; Zhang *et al.*, 1995a) and electrophysiological responses from axotomized DRG neurons (Abdulla & Smith, 1999).

Immunohistochemical studies indicate NPY is present in fibres and varicosities in the superficial laminae (I-III) of the spinal cord (Doyle & Maxwell, 1994; Hokfelt *et al.*, 1981; Hunt *et al.*, 1981; Krukoff, 1987). These fibres form axo-axonic synapses in cat spinal cord (Doyle & Maxwell, 1993) and in the lamprey these fibres synapse onto primary afferent fibres (Bongianni *et al.*, 1990; Parker *et al.*, 1998). NPY mRNA is also present in the dorsal horn (Zhang *et al.*, 1994a) and appears to be expressed in interneurons in rat spinal cord (Minson *et al.*, 2001).

The presence of NPY or NPY mRNA in the dorsal horn is unaffected by acute transection of the dorsal roots or peripheral nerves (Gibson *et al.*, 1984) suggesting NPY originates from intrinsic sources in the spinal cord or from supraspinal projections. A small number of NPY-immunoreactive (NPY-IR) neurons in the locus coeruleus (Holets *et al.*, 1988) and the rostral ventrolateral medulla (Blessing *et al.*, 1987) send their axons to the spinal cord, although they do not appear to form synapses in the dorsal horn. However, in the dorsal horn, application of colchicine, a blocker of axonal transport, causes large increases in NPY-IR neurons in normal rats and in rats that have peripheral nerve injury (Cougnon-Aptel *et al.*, 1999; Hunt *et al.*, 1981; Sasek & Elde, 1985). These NPY-IR neurons are found predominantly in lamina II, but cells are also present in laminae I and III and are described as being medium to large in size (de Quidt & Emson, 1986; Hunt *et al.*, 1981).

As mentioned above, neurons in the superficial dorsal horn contain GABA and glycine and often these transmitters are colocalized. Interestingly, NPY-IR is restricted primarily to GABAergic neurons which do not use glycine (Laing *et al.*, 1994; Rowan *et al.*, 1993). Moreover, NPY-IR does not colocalize with [Met]enkephalin-containing cells (Rowan *et al.*, 1993). Similarly in lamprey, NPY- and GABA-IR are colocalized in dorsal horn neurons (Parker *et al.*, 1998). In lamina III, GABAergic neurons, that also contain NPY, synapse onto the dendrites of cells that express the NK-1 receptor (Polgar *et al.*, 1999). NPY binding sites are present in the superficial dorsal horn, with the highest density in lamina I and II (Kar & Quirion, 1992; Martel *et al.*, 1990). After neonatal capsaicin treatment or dorsal rhizotomy these binding sites are decreased (Kar & Quirion, 1992) suggesting a certain proportion of these receptors are located on primary afferent fibres. Studies using [^{125}I]peptide YY (PYY) as ligand suggest these binding sites are Y2 receptors which are dramatically up-regulated following peripheral axotomy (Zhang *et al.*, 1995a). Y1 receptor immunoreactivity or mRNA is present in neurons in lamina II (Ji *et al.*, 1994; Migita *et al.*, 2001; Zhang *et al.*, 1999) with higher levels in the inner portions of lamina II (Zhang *et al.*, 1994a). These receptors are localized primarily on the dendrites of somatostatin-immunoreactive neurons that receive input from Y1 receptor-negative axon terminals, suggesting that the Y1 receptor is a postsynaptic receptor in the dorsal horn (Zhang *et al.*, 1999). Recent studies indicate presynaptic Y1 receptors may be present in the dorsal horn (Bao *et al.*, 2002; Brumovsky *et al.*, 2002). Following peripheral nerve inflammation, levels of Y1 receptor mRNA are up-regulated in cells located in the medial portions of laminae II and III (Ji *et al.*, 1994).

In spite of considerable anatomical data regarding the distribution of NPY in the dorsal horn, little is known about its functional role in this region. Studies using the antibody microprobe technique show that stimulation of unmyelinated sensory fibres triggers the release of immunoreactive-Substance P (IR-Substance P) in laminae I and II (Duggan *et al.*, 1988b; Duggan *et al.*, 1988a). Microinjection of NPY into laminae I and II leads to a decrease in IR-Substance P (Duggan *et al.*, 1991). This technique has been extended to examine the release of immunoreactive-NPY (IR-NPY) in lamina I and II (Mark *et al.*, 1997). This study indicates that there is extensive basal release of IR-NPY in the dorsal horn that likely arises from intrinsic sources because it is unaffected by spinal cord transection or peripheral nerve stimulation. By contrast, in nerve-injured animals, electrical stimulation of large diameter sensory fibres leads to increased IR-NPY throughout the dorsal horn (Mark *et al.*, 1998). This finding is consistent with *in situ* hybridization studies that show NPY is preferentially contained in large diameter DRG neurons following peripheral nerve injury (Noguchi *et al.*, 1993).

In lamprey spinal cord, NPY and the related peptide, PYY, reduce the amplitude of monosynaptic EPSPs evoked by dorsal root stimulation (Parker *et al.*, 1998). This effect on EPSP amplitude is not accompanied by a change in input resistance or membrane potential, nor does it affect depolarizations evoked by exogenous glutamate. This suggests a presynaptic action of NPY. In approximately 50% of the neurons in lamprey dorsal horn, NPY reduces the duration of presynaptic action potentials. The $GABA_B$ agonist, baclofen also acts presynaptically to reduce EPSP amplitude (Christenson & Grillner, 1991; Parker *et al.*, 1998) and this effect is potentiated by NPY, suggesting NPY and $GABA_B$ receptors are colocalized on primary afferent terminals. NPY also affects motor neuron excitability and ventral root activity in response to sensory stimulation, presumably by acting on primary afferent fibres that synapse onto motor neurons (Ullström *et al.*, 1999).

The actions of NPY in the DRG and dorsal horn suggest it may exert antinociceptive actions in the spinal cord. Intrathecally or intracerebroventricularly administered NPY produces a strong antinociceptive effect in both normal and axotomized rats (Broqua *et al.*, 1996; Hua *et al.*, 1991; Xu *et al.*, 1994). This effect of NPY appears to be mediated by a Y1 receptor in normal rodents (Broqua *et al.*, 1996) and by both Y1 and Y2 receptors in axotomized rodents (Xu *et al.*, 1999). Recently, Y1 receptors, but not Y2 receptors, have been shown to produce an antihyperalgesic effect in inflammatory pain (Taiwo & Taylor, 2002). Interestingly, in mice lacking the Y1 receptor NPY does not produce analgesia (Naveilhan *et al.*, 2001). Furthermore, these mice have reduced antinociception to painful thermal, chemical and mechanical stimuli, as well as increased neuropathic pain.

In summary, NPY receptor activation produces analgesia which appears to be mediated, to some degree, at the level of the spinal cord, although this finding is controversial. Binding and immunocytochemical studies show NPY Y2 receptors are present on primary afferent fibres which terminate in the substantia gelatinosa, whereas local dorsal horn interneurons appear to express postsynaptic Y1 receptors. Activation of NPY receptors suppresses excitatory synaptic transmission in the dorsal horn and decreases transmitter release, actions that are shared by opioids. The similar cellular actions of NPY are important for the management of neuropathic pain, as μ-opioid receptor expression decreases in the DRG and dorsal horn after nerve injury, whereas NPY and NPY receptor expression increases after nerve injury. This suggests NPY may be effective for the management of neuropathic pain.

Nociceptin

Molecular cloning of the μ-, δ- and κ-opioid receptors led to the discovery of a novel GPCR, identified as opioid-like receptor 1 (ORL$_1$ or OP4) (Dhawan *et al.*, 1996; Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), whose putative ligand is the heptadecapeptide nociceptin/orphanin FQ (N/OFQ). Although N/OFQ appears to have analgesic actions in the spinal cord (Hao *et al.*, 1998; Henderson & McKnight, 1997) it may induce hyperalgesia when injected supraspinally (Reinscheid *et al.*, 1995). However, it should be noted that this initial observation of N/OFQ-induced hyperalgesia (Reinscheid *et al.*, 1995) has been questioned (Henderson & McKnight, 1997) and may be due to attenuation of stress-induced analgesia (Grisel & Mogil, 2000). Despite this, the cellular actions of N/OFQ resemble those of 'classic' analgesic opioid agonists, that is, suppression of Ca^{2+} channel current (I_{Ca}), activation of an inwardly-rectifying K^+ conductance and suppression of neurotransmitter release.

N/OFQ, like μ- and κ-opioids, inhibits high-voltage activated (HVA) I_{Ca} (primarily N-type I_{Ca}) in DRG (Abdulla & Smith, 1997b) and trigeminal ganglion neurons (Borgland *et al.*, 2001). The effects of N/OFQ and μ-opioid agonists are most pronounced in small DRG neurons, which are putative nociceptors (Bessou & Perl, 1969). The action of N/OFQ on HVA I_{Ca} involves a G-protein-dependent mechanism, as N/OFQ produces marked changes in calcium conductance (g_{Ca}) activation kinetics and the suppressive effects of N/OFQ are relieved by strong depolarization (Abdulla & Smith, 1997b).

As previously discussed, opioids, such as morphine, appear to have little clinical efficacy in the management of neuropathic pain. By contrast, N/OFQ becomes more effective at suppressing N-type I_{Ca} in DRG neurons following axotomy of the sciatic nerve (Abdulla & Smith, 1998). Furthermore, in animal models of neuropathic pain N/OFQ appears to alleviate the hyperalgesia and allodynia associated with peripheral nerve injury and inflammation (Hao *et al.*, 1998; Yamamoto *et al.*, 1997). These findings are of particular interest as they suggest a potential clinical role for ORL_1-selective agonists in the management of neuropathic pain.

As mentioned earlier, the analgesic actions of opioids in the spinal cord involve presynaptic and postsynaptic mechanisms, such as reduced transmitter release (Glaum *et al.*, 1994; Grudt & Henderson, 1998; Jessell & Iversen, 1977; Kohno *et al.*, 1999; Yaksh *et al.*, 1980) and activation of an inwardly-rectifying K^+ conductance (Grudt & Williams, 1993; Grudt & Williams, 1994). N/OFQ appears to have a general inhibitory action in the dorsal horn of the spinal cord, which is reminiscent of the actions of μ-, δ- and κ-opioid agonists.

Repetitive stimulation of afferent nociceptive C-fibres invokes wind-up (see Table 1-1). This involves an increase in the number of action potentials generated by a dorsal horn neuron after each successive stimulus during a pulse train (Mendell, 1966). Intrathecal application of N/OFQ to dorsal horn neurons appears to modulate nociceptive responses by selectively reducing C-fibre-evoked wind-up and post-discharge action potential activity following repetitive stimulation (Stanfa *et al.*, 1996). In neonatal rat spinal cord, N/OFQ depresses glutamatergic ventral root potentials evoked by dorsal root stimulation in a concentration-dependent manner (Faber *et al.*, 1996). This action does not appear to involve μ, δ or κ-opioid receptors, as the effects of N/OFQ are not blocked by the non-selective opioid receptor antagonist, naloxone (Faber *et al.*, 1996).

N/OFQ suppresses evoked EPSPs/EPSCs and reduces the frequency of mEPSCs in substantia gelatinosa neurons (Lai *et al.*, 1997; Luo *et al.*, 2002). This synaptic depression is not accompanied by changes in input resistance or resting membrane potential (Lai *et al.*, 1997). Also, N/OFQ has no effect on depolarizations evoked by exogenously applied glutamate (Lai *et al.*, 1997). Therefore, its actions appear to be predominantly presynaptic. Surprisingly, N/OFQ is more effective at suppressing C-fibre-evoked EPSCs than to Aδ-fibre-evoked EPSCs (Luo *et al.*, 2002). Similarly, in lamina I neurons, N/OFQ reduced the amplitude of evoked EPSCs (Liebel *et al.*, 1997) and decreased the frequency, but not the amplitude, of mEPSCs, and had no effect on glutamate-evoked currents, all of which suggest a presynaptic site of action (Liebel *et al.*, 1997). Of note, N/OFQ appears to selectively suppress EPSCs in the dorsal horn without affecting inhibitory synaptic transmission (Ahmadi *et al.*, 2001; Lai *et al.*, 1997; Liebel *et al.*, 1997; Luo *et al.*, 2002; Moran & Smith, 2002; Zeilhofer *et al.*, 2000). Similar to μ-, δ- and κ-opioid agonists, N/OFQ also activates an inwardly-rectifying K^+ conductance in medullary (Jennings, 2001) and spinal cord dorsal horn neurons (Luo *et al.*, 2001; Moran & Smith, 2002).

Other reports, however, using slightly different preparations of spinal neurons suggest that both N/OFQ and opioids can exert postsynaptic effects by modulating glutamate responses. For example, in acutely dissociated dorsal horn neurons, N/OFQ has been shown to inhibit glutamate-, kainic acid- and quisqualic acid-evoked currents (Shu *et al.*, 1998). This is similar to the actions of μ-opioid agonists that inhibit AMPA-activated currents in dissociated dorsal horn neurons (Kolaj & Randic, 1996).

In vivo extracellular recordings from medullary dorsal horn (trigeminal nucleus caudalis) neurons show N/OFQ inhibits responses evoked by AMPA and NMDA in nociceptive and non-nociceptive neurons (Wang *et al.*, 1996) in a manner similar to 'classic' opioids (Zhang *et al.*, 1996b). N/OFQ also inhibits Substance P-induced facilitation of NMDA-evoked responses in medullary dorsal horn (Wang *et al.*, 1999).

The Role of Nociceptin and Opioids in Supraspinal Pathways

Periaqueductal Grey

The midbrain periaqueductal grey matter (PAG) is subdivided into dorsolateral, dorsomedial, lateral and ventrolateral regions. Neurons in the ventrolateral PAG are of special interest as they project to the rostral ventromedial medulla (RVM) and thence to the dorsal horn of the spinal cord (Basbaum & Fields, 1984; Osborne *et al.*, 1996).

Opioids are thought to exert an antinociceptive effect by disinhibiting neurons in the ventrolateral PAG which project to the RVM (Osborne *et al.*, 1996). Although N/OFQ and μ-opioids affect the same set of ionic conductances throughout the PAG, the distributions of their effects are not identical. For example, in the PAG as a whole, N/OFQ activated an inwardly-rectifying K^+ conductance in all neurons, whereas [Met]enkephalin is effective on only 60% of the neurons (Vaughan *et al.*, 1997). The distribution of the effects of N/OFQ is therefore more similar to those of the $GABA_B$ agonist, baclofen, than to those of a 'classic' opioid.

In ventrolateral PAG neurons, N/OFQ inhibits evoked IPSCs in most cells and thereby produces disinhibition in a similar fashion to μ-opioids (Vaughan *et al.*, 1997). Because it also attenuates EPSCs in this region and promotes membrane hyperpolarization, the disinhibitory effects of N/OFQ may be blunted by concurrent inhibitory actions. N/OFQ-induced inhibition of synaptic events is also associated with a reduction in the frequency of TTX-insensitive mIPSCs and mEPSCs (Vaughan *et al.*, 1997) suggesting a presynaptic inhibitory effect on transmitter release. This interpretation is also supported by the observations that attenuation of synaptic responses is accompanied by an increase in paired-pulse facilitation (Vaughan *et al.*, 1997).

In acutely dissociated PAG neurons, N/OFQ inhibits N-type and P/Q-type I_{Ca}, while having little effect on L-type or R-type I_{Ca} (Connor & Christie, 1998). These effects are blocked by naloxone or the inactive N/OFQ analogue des[Phe^1]-nociceptin (Connor & Christie, 1998). The inhibition of I_{Ca} in PAG neurons is likely mediated via a G-protein βγ-subunit signaling pathway, as indicated by a slowing of g_{Ca} activation kinetics (Connor & Christie, 1998). Interestingly, N/OFQ appears to be more effective at inhibiting I_{Ca} in the PAG than either [Met]enkephalin or the μ-opioid agonist, DAMGO (Connor & Christie, 1998).

Rostral Ventromedial Medulla

As described above, the PAG has projections to the RVM, a region important for modulating nociceptive transmission. In the RVM, two cell types are thought to be involved in pain modulation and have different responses to opioids (Fields *et al.*, 1991). N/OFQ has an anti-opioid action in the RVM (Heinricher *et al.*, 1997; Pan *et al.*, 2000). N/OFQ inhibits the firing of both on- and off-cells in the RVM. However, when N/OFQ is co-applied with DAMGO, opioid-induced disinhibition of the off-cells is prevented. It is likely that N/OFQ directly inhibits the off-cell AP firing rate, thus preventing opioid-induced disinhibition and thereby increasing nociception.

Nocistatin

Recent studies have identified and characterized the actions of nocistatin (NST), a peptide derived from the N/OFQ precursor peptide, prepronociceptin (Okuda-Ashitaka *et al.*, 1998; Okuda-Ashitaka & Ito, 2000). The actions of NST are controversial. In some studies it appears to antagonize N/OFQ-induced allodynia and hyperalgesia (Okuda-Ashitaka *et al.*, 1998; Okuda-Ashitaka & Ito, 2000) while in other studies it causes hyperalgesia and antagonizes N/OFQ-mediated analgesia (Zeilhofer *et al.*, 2000). NST selectively suppresses the release of GABA and glycine in the substantia gelatinosa via a presynaptic mechanism (Ahmadi *et al.*, 2001; Zeilhofer *et al.*, 2000) which may explain its hyperalgesic effect. Also, in locus coeruleus neurons NST is ineffective at preventing N/OFQ-induced inhibition of I_{Ca} (Connor *et al.*, 1999).

Morphine-3β-D-glucuronide

As discussed earlier, some studies suggest that administration of N/OFQ may cause allodynia and hyperalgesia (Okuda-Ashitaka *et al.*, 1998; Okuda-Ashitaka & Ito, 2000; Reinscheid *et al.*, 1995). These actions of N/OFQ are similar to those of the morphine metabolite, morphine-3β-D-glucuronide (M3G) (Woolf, 1981; Yaksh *et al.*, 1986; Yaksh & Harty, 1988). Like N/OFQ, M3G does not bind to μ-, δ- or κ-opioid receptors (Lambert *et al.*, 1993; Löser *et al.*, 1996; Pasternak *et al.*, 1987). We initially hypothesized M3G may be an agonist at the ORL_1 receptor. In comparing the effects of M3G with μ-opioids and N/OFQ some new information regarding the spinal actions of all three substances were obtained (Moran & Smith, 2002).

Hypothesis

The previous sections have compared the neurophysiological actions of opioid peptides with those of other neuropeptides present in the dorsal horn. Opioids have limited efficacy in the management of neuropathic pain. This may be attributed to down-regulation of their receptors on DRG neurons and in the dorsal horn after peripheral nerve injury. Therefore, other spinal neuropeptides represent potential targets for the treatment of neuropathic pain. However, antagonism of the actions of excitatory neuropeptides, such as Substance P, is generally ineffective for the treatment of neuropathic pain. In view of this, I propose: agents that mimic the effects of spinal inhibitory neuropeptides will be effective analgesics in neuropathic pain states.

First, I have considered investigating the spinal actions of NPY, as its documented effects on most neurons (inhibition of N-type Ca^{2+} channel current, decreased neurotransmitter release and activation of GIRK channels) resemble those of opioids. These similar actions are summarized in Figure 1-3. Furthermore, while DRG and dorsal horn opioid receptors are down-regulated after nerve injury, NPY receptors, especially Y2 receptors, are up-regulated following nerve injury.

Second, I have considered testing the effect of agents that may mimic the actions of nociceptin. In view of the unfavourable pharmacokinetic properties of neuropeptides, I have examined the actions of the morphine metabolite, M3G.

Thus, the two main hypotheses to be addressed are:

1. NPY has opioid-like actions in the dorsal horn of the spinal cord.
2. M3G stimulates ORL_1 receptors and has nociceptin-like effects in the dorsal horn of the spinal cord.

Table 1-1. Pain definitions and terminology.

Type of Pain	Definition
Acute Pain	Elicited by tissue damage that in turn activates nociceptive transducers. This type of pain lasts for a relatively finite time and generally remits when the underlying pathology resolves.
Chronic Pain	Usually elicited by an injury and extends for a long period of time. Often the underlying pathology does not explain the presence or extent of the pain. Treatment is rarely effective.
Allodynia	Pain evoked by an innocuous or non-painful stimulus.
Central Pain	Pain induced or caused by a lesion to the central nervous system.
Central Sensitization	An increase in the excitability and responsiveness of neurons in the spinal cord.
Hyperalgesia	An increased responsiveness to a stimulus that is normally painful.
Neuropathic Pain	Pain initiated or caused by a lesion or dysfunction in the nervous system
Wind-up/second pain	A slow, temporal summation caused by repetitive stimulation (>1Hz) of C-fibres resulting in increased excitability. It is often perceived as a gradual increase in the magnitude of the pain.

Table 1-2. Clinical aspects of neuropathic pain.

Abnormal quality: burning, prickling, stinging
Paroxsymal pain
Sensory impairment
Allodynia and hyperalgesia
Abnormal sympathetic function
Immediate or delayed onset of pain after injury

Table 1-3. NPY Receptor Subtypes.

Receptor	**Y1**	**Y2**	**Y4**	**Y5**	**Y6**
Agonist Order of Potency	**NPY ≥ PYY>> PP**	**NPY ≅ PYY >> PP**	**PP > NPY≅ PYY**	**NPY ≥ PYY ≥ PP**	**NPY ≅ PYY > PP**
Selective Agonists	**[Phe^{7},Pro^{34}]NPY**	**[Ahx$^{5-24}$]NPY**	**PP**	**[chicken PP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Q^{34}]human PP)**	**None**
Preferring Agonists	**[Pro^{34}]NPY** **[Pro^{31}]PYY** **[Leu^{31},Pro^{34}]NPY** **[Leu^{31},Pro^{34}]PYY**	**NPY(3-36)** **PYY(3-36)** **NPY(13-36)** **PYY(13-36)**			
Antagonists	**BIBP3226** **BIBO3304**	**BIIE0246**	**none**	**CGP71683A**	**None**
Signal Transduction Mechanisms	**G_i/G_o,** **↓ adenylyl cyclase**	**G_i/G_o** **↓ adenylyl cyclase**	**G_i/G_o** **↓ adenylyl cyclase**	**G_i/G_o** **↓ adenylyl cyclase**	**?** **↓ adenylyl cyclase**

Figure 1-1. Primary afferent inputs to laminae I and II. Laminae I and II are major sites of termination for unmyelinated C-fibres (shown in black) and thinly myelinated Aδ fibres (shown in blue). C-fibres are polymodal nociceptors and respond to intense thermal, mechanical and chemical stimuli. Aδ-fibres respond to intense mechanical stimulation and noxious heat. Conduction velocity of C-fibres is < 2m/s and Aδ-fibres 5-55 m/s.

Figure 1-1

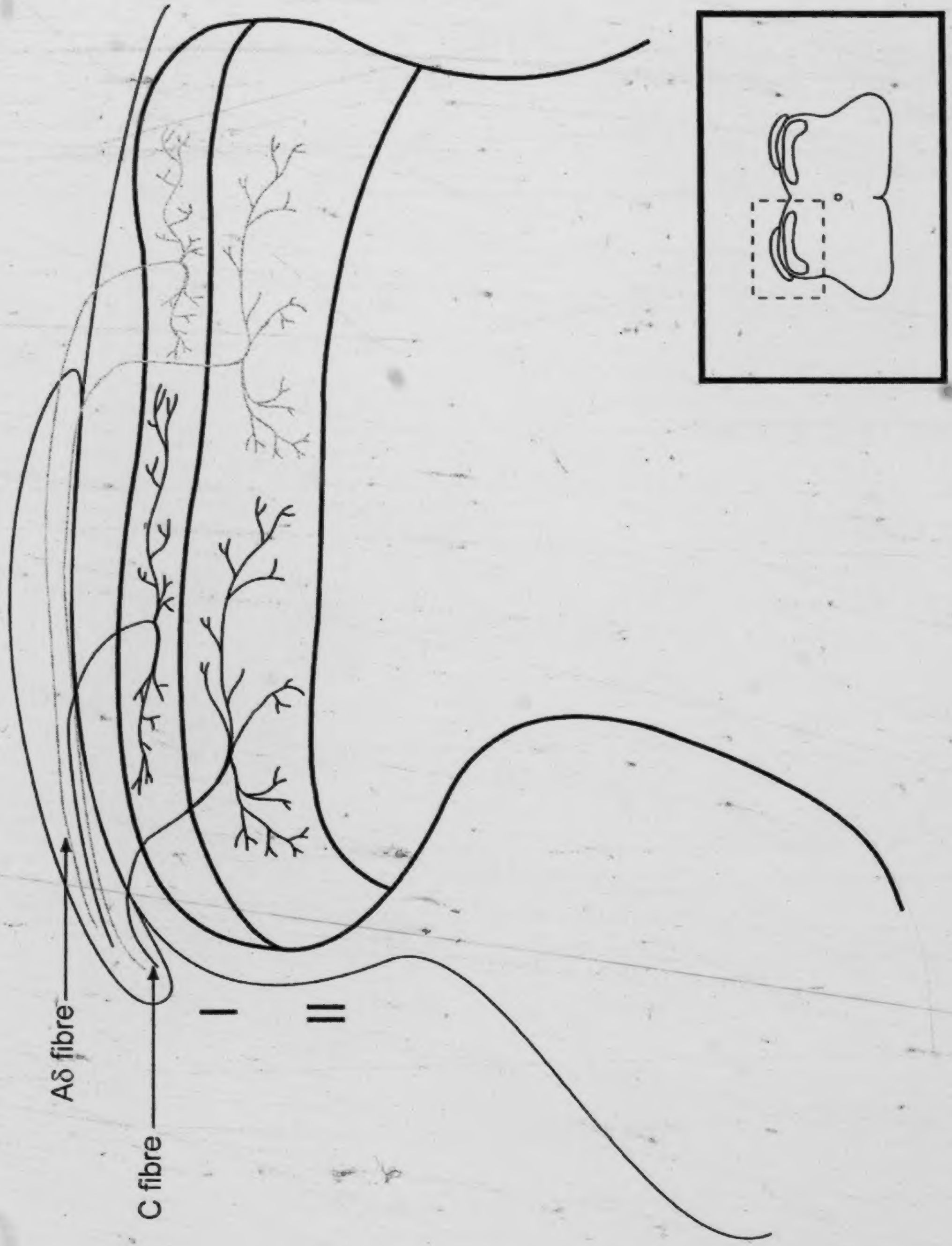

Aδ fibre
C fibre
I
II

Figure 1-2. Diagram of the neuronal circuitry of laminae I and II. Lamina I contains at least four different cells types: (1) fusiform spiny neurons; (2) pyramidal neurons; (3) flattened aspiny neurons; and (4) multipolar neurons. Lamina II contains at least five different cell types. For clarity, only islet cells and stalked cells are shown. Islet cells are likely inhibitory interneurons and stalked cells are likely excitatory interneurons.

Figure 1-2

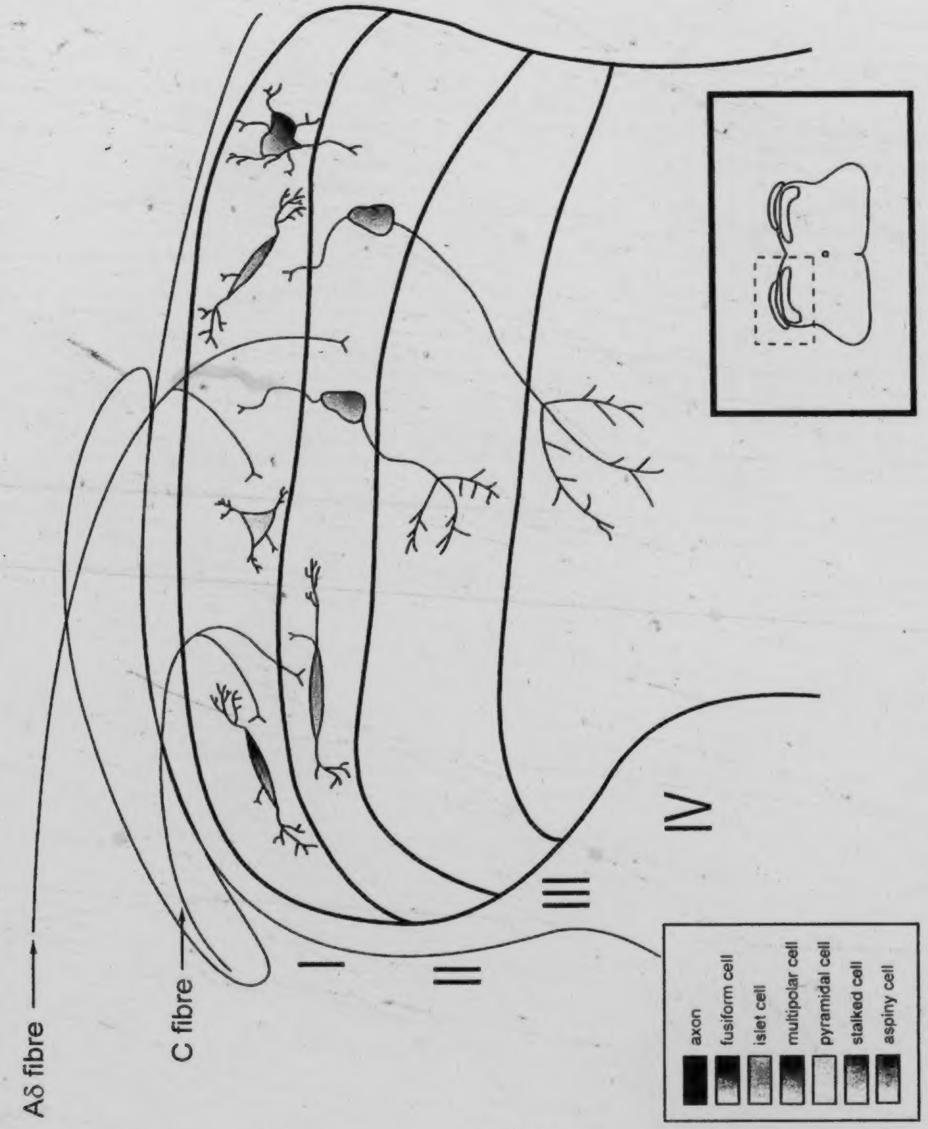
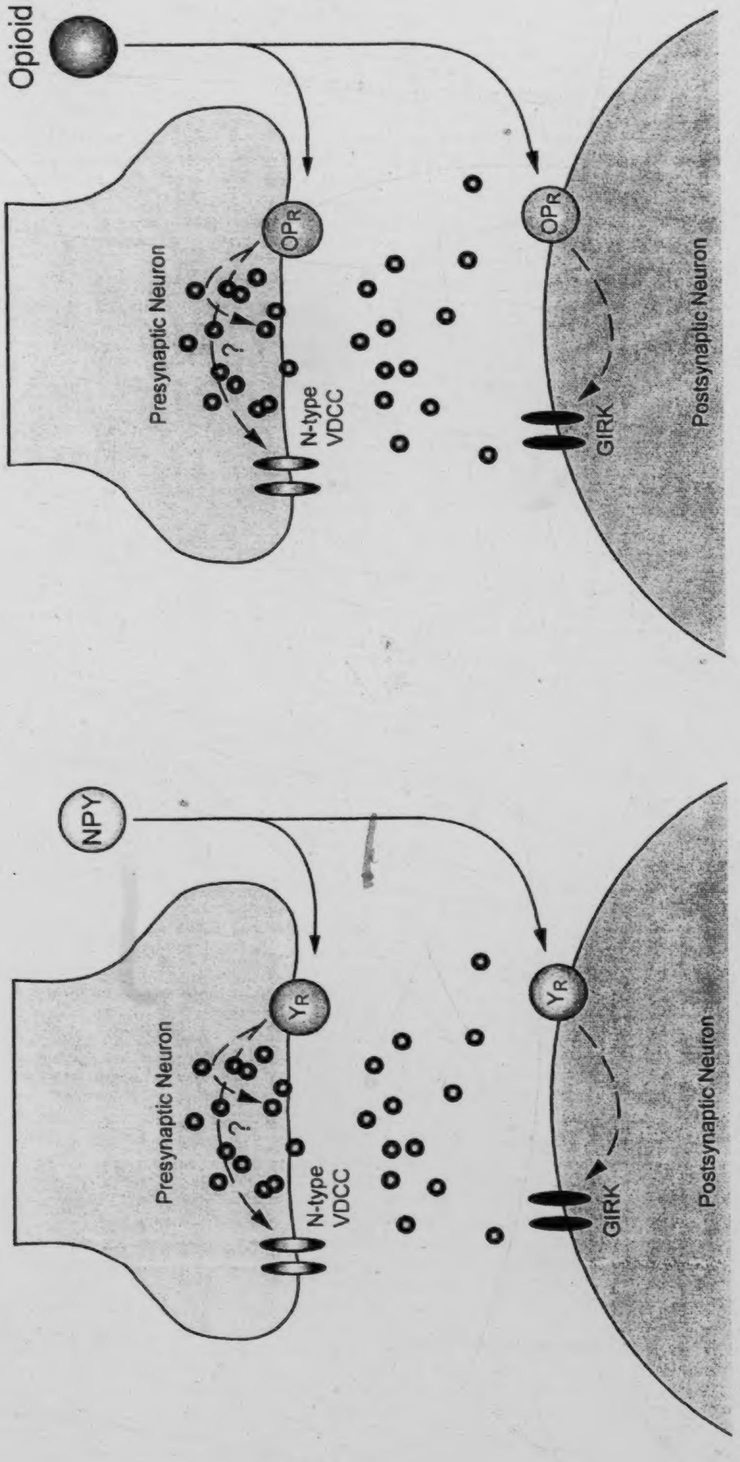

Aδ fibre
C fibre
I
II
III
IV
axon
fusiform cell
islet cell
multipolar cell
pyramidal cell
stalked cell
aspiny cell

Figure 1-3. Diagram illustrating the similar neurophysiological actions of neuropeptide Y and opioids. NPY and opioids suppress N-type Ca^{2+} channels, decrease neurotransmitter release and activate G-protein-coupled inwardly-rectifying K^+ channels (GIRK channels). Legend: OP_R (opioid receptor); Y_R (NPY receptor).

Figure 1-3


Opioid
OPR
Presynaptic Neuron
?
N-type VDCC
GIRK
Postsynaptic Neuron
NPY
YR
Presynaptic Neuron
?
N-type VDCC
GIRK
Postsynaptic Neuron

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Chapter 2

General Methods: Spinal Cord Slice Preparation and Whole-Cell Patch-Clamp Recordings from Visually Identified Substantia Gelatinosa Neurons

Methods

Spinal Cord Slice Preparation

All procedures were carried out in compliance with the guidelines of the Canadian Council for Animal Care, the University of Alberta Health Sciences Laboratory Animal Services Welfare Committee and the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Sprague-Dawley rats (14-42 d) were deeply anesthetized with urethane (1.5 g/kg, i.p.). Rats were placed dorsal side up and an incision was made was made through the skin with surgical scissors (14002-13, Fine Science Tools Vancouver BC, Canada). The incision extended from the base of the tail to the skull. The skin was detached from the underlying muscle by blunt dissection. By grasping the vertebral column at the mid-thoracic region with Adson forceps (11027-12, Fine Science Tools) a vertically-oriented cut was made through the vertebral column. The muscle overlying the vertebral column was grasped with Adson forceps and lifted gently upwards in order to view the spinal cord within the vertebral column. The incision site was irrigated using a Pasteur pipette with a small amount (~2 ml) of ice-cold oxygenated (95% O_2-5% CO_2) dissection solution containing (in mM): 118 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, 1.5 $CaCl_2$, 5 $MgCl_2$, 25 D-glucose, 1 kynurenic acid. A laminectomy was performed by inserting the tips of spring scissors (15012-12, Fine Science Tools) between the ventral horn and underlying vertebrae. The laminectomy extended from the thoracic to the sacral portions of the spinal cord. To prevent damage to the spinal cord, the tips of the spring scissors were pointed downward toward the underlying bone of the vertebral column. The spinal cord was removed *en bloc,* with the overlying muscle and dorsal vertebrae still attached, and transferred to a Sylgard-coated (Dow-Corning, USA) 60 mm glass petri dish (Corning, USA) containing ice-cold oxygenated dissection solution (see above). The tissue was pinned, with the ventral horn of the spinal cord exposed, to the bottom of the petri dish by inserting pins through the muscle on either side of the vertebral column. This is illustrated schematically in Figure 2-1.

With the aid of a dissection microscope, the spinal cord was carefully removed from the vertebral column. The dura mater at the rostral part of the cord was gently grasped with watchmaker forceps (11251-10, Fine Science Tools) and the spinal cord was lifted upwards by 3-5 mm. The dorsal and ventral roots were cut using small spring scissors (15024-10, Fine Science Tools) and the spinal cord was lifted out of the vertebral column. The spinal cord was positioned with the dorsal horn facing up and the dura mater was removed. All ventral roots were cut near their exit zones with small spring scissors. The dorsal roots were cut near their entry zones, with the exception of the L4-L5 dorsal roots.

The spinal cord was glued with cyanoacrylate glue to a 4% agar block cut in the shape of a trapezoid. The agar block, with attached spinal cord, was glued to the bottom of a 60 mm glass petri dish, submerged in ice-cold dissection solution and continuously bubbled with 95%O_2-5% CO_2 oxygenated dissection solution. Transverse slices (300-500 μm) were cut using a Vibratome (TPI, USA). To prevent the tissue from being pushed or deformed, the blade advance speed was set between 1-4 mm/min and the widest blade excursion possible was used (~1.25 mm/sec) (Edwards, 1995). The setup for preparing transverse spinal cord slices is shown in Figure 2-2. The slow advance speed was achieved by inserting a 100 Ω resistor at the rheostat control for the blade advance speed. The blade advance speed was calibrated by measuring the distance the blade advanced in a 1 min interval for various speed settings. During slicing the blade advance speed was constantly monitored and changed according to the progress through the tissue. Slices were incubated in a holding chamber (Sakmann & Stuart, 1995) at 36°C for 1 h and stored at room temperature (~22°C) in oxygenated, dissection solution (see above, without 1 mM kynurenic acid), unless specified otherwise. A diagram of the slice storage chamber is shown in Figure 2-3.

Visualization of Substantia Gelatinosa Neurons Using Infrared-Differential Interference Contrast Microscopy

The following procedures were adapted from previous studies (Sakmann & Stuart, 1995; Stuart *et al.*, 1993). Spinal cord slices were viewed with a Zeiss Axioskop FS equipped with a 4X 0.10-numerical aperture (NA) objective (Achroplan, Zeiss Canada, Toronto, ON, Canada), a 40X 0.75-NA water immersion objective, a 0.9-NA condenser and DIC optics. Illumination was provided from a 100 W halogen lamp and a 12 V power supply. The optical setup for IR-DIC microscopy is shown in Figure 2-4 and Figure 2-5. Slices were initially viewed with the 4X objective to position stimulating electrodes. The substantia gelatinosa was identifiable as a translucent band across the dorsal horn. Using the 40X water immersion objective the substantia gelatinosa was viewed with visible light through the oculars and the surface of the slice was brought into focus. The microscope was set-up for optimal Köhler illumination. Briefly, the field diaphragm was closed to ~2/3 of the field of view and the diaphragm image was focused by slightly raising or lowering the condenser. The field diaphragm was opened until it just disappeared from the field of view. The IR filter (λ = 750 nm Zeiss Canada) was inserted into the light path prior to the DIC polarizer and the light was increased to maximum intensity. The light path was switched to the IR-sensitive video camera (NC-70, Dage-MTI, Michigan City, IN, USA) and viewed on a video monitor. Additional magnification was achieved by placing a 4X magnification tube between the microscope and the IR-sensitive camera. The image contrast was enhanced by adjusting the DIC slider and the black level and gain controls on the camera control unit. 'Healthy' cells had a 'smooth' appearance, were not swollen and easily dimpled by the patch pipette. 'Unhealthy' cells typically had a high-contrast membrane, appeared swollen or had a 'wrinkled' appearance. Cells could be visualized to a depth of up to 100 μm. However, this distance typically decreased to 40-50 μm as the animals became older (> 28 d).

Arrangement of Spinal Cord Slices for Patch-Clamp Recordings

The slice-recording chamber was mounted on a fixed-stage and the microscope was attached to two linear actuators (Model 433, Newport, Irvine, CA, USA). This allowed the position of the microscope to be changed without affecting the relative position of the recording and stimulating electrodes. The manipulator (WR-88, Narishige, Tokyo, Japan) for positioning stimulating electrodes was attached directly to the fixed-stage. The manipulator for the recording electrode (MP285, Sutter Instruments, Novato, CA, USA) was attached to a column (Model 200, Newport, Irvine, CA, USA) that was mounted directly on the anti-vibration table. The approach angle for the recording and stimulating electrodes was approximately 25° to horizontal.

A single spinal cord slice was placed into a circular glass-bottomed (#1 thickness, Fisher Scientific, Toronto, ON, Canada) recording chamber. The recording chamber had a diameter of 25 mm and volume of ~1 ml. The spinal cord slice was oriented with the substantia gelatinosa towards the back of the microscope. The slice was held in place with a U-shaped platinum wire frame with attached parallel nylon threads, ensuring no threads were overlying the substantia gelatinosa. The U-shaped frame was made by flattening ~2 cm of 0.5 mm diameter platinum wire (Goodfellow, Berwyn, PA, USA) in a vise. The nylon threads were obtained from nylon stockings. A small section of stocking was tightly stretched over the top of a 100 ml beaker and held in place with an elastic band. A small hole was made in the stocking which resulted in an array of parallel compound threads separated from each other by ~1-2 mm. Typically these compound threads were made of 6-8 individual strands. With the aid of a dissecting microscope, these threads were separated into individual strands by grasping a single strand with watchmaker forceps and cutting the strand. ,. The platinum wire frame was then coated with cyanoacrylate glue and placed on top of the nylon threads. To set the glue more rapidly, distilled water was poured over the frame. After drying for ~30 min the frame was cut away from the remaining nylon stocking.

Patch-Clamp Recording and Stimulation of Spinal Cord Slices

Spinal cord slices were transferred to the recording chamber and superfused (flow rate ~1-2 ml/min) at room temperature (~22°C) with 95% O_2-5% CO_2 saturated aCSF (in mM): 127 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 25 D-glucose, pH 7.4. For recording excitatory postsynaptic currents (EPSCs), bicuculline (10 μM) and strychnine (1 μM) were included to block inhibitory synaptic inputs. For recording inhibitory postsynaptic currents (IPSCs), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and DL-2-Amino-5-phosphonovaleric acid (DL-AP5; 50 μM) were included to block excitatory synaptic inputs. Tetrodotoxin (TTX; 1μM) was included when recording miniature EPSCs and IPSCs (mEPSC; mIPSC).

Whole-cell recordings were made with an npi SEC 05L amplifier (npi Electronic, Tamm, Germany) in discontinuous single-electrode voltage-clamp or bridge-balance current-clamp mode. Recordings involved either the 'blind' whole-cell patch-clamp technique (Blanton *et al.*, 1989) or visually-identifying individual substantia gelatinosa neurons using infrared-differential interference contrast (IR-DIC) video microscopy (Dodt & Zieglgansberger, 1990; Sakmann & Stuart, 1995; Stuart *et al.*, 1993).

Patch pipettes were pulled from thin-walled borosilicate glass (1.5 mm o.d., 1.12 mm i.d.; TW-150F-4, WPI, Sarasota, FL, USA). Pipettes for recording APs and postsynaptic K^+ currents had resistances of 5-10 MΩ when filled with an internal solution containing (in mM): 130 potassium gluconate, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP, pH 7.2, 290-300 mOsm. For recording synaptic currents, a Cs^+-based internal solution containing (in mM): 140 CsCl, 5 HEPES, 10 EGTA, 2 $CaCl_2$, 2 Mg-ATP, 0.3 Na-GTP, pH 7.2, 290-300 mOsm was used.

When recording EPSCs and IPSCs, the voltage-gated ion channel blocker QX-314 (5 mM) was included in the internal solution to prevent action potential discharge.

QX-314 was omitted from the internal solution when recording APs and postsynaptic K^+ currents. For voltage-clamp experiments, membrane potential was clamped at –60 or –70 mV for recording EPSCs and IPSCs when a CsCl-based internal was used. The E_{Cl} was ~0 mV therefore IPSCs appeared as inward currents at a holding potential of –60 or –70 mV. When a K^+-gluconate-based internal solution was used membrane potential was clamped at –60 or –70 mV for recording EPSCs and postsynaptic K^+ currents and 0 mV for recording IPSCs. Switching frequencies were typically between 30-40 kHz. Signals were filtered at 1-2 kHz and digitized between 5 and 10 kHz. These filter and sampling frequencies were selected to ensure that the Nyquist theorem, which states that the sampling frequency should be at least twice the filter frequency, was not violated.

EPSCs were evoked at 0.05 Hz with a bipolar concentric stimulating electrode (FHC, Bowhoidon, ME, USA) or a custom-made bipolar, Teflon-coated nichrome stimulating electrode These were placed on the dorsal root or near the dorsal root entry zone to activate primary afferent fibres. To manufacture the Teflon-coated nichrome stimulating electrodes, two 20 cm strands of nichrome wire (7620, A-M Systems, Carlsborg, WA, USA) were inserted into a 10 cm thin-walled borosilicate glass tube (WPI, Sarasota, FL, USA) with approximately 1 cm of wire protruding from one end of the glass tubing. A small amount of epoxy was applied to each end of the glass tubing to seal the wires. After the epoxy cured, the 1 cm strands were twisted together. The ends were cut with a scalpel blade to expose the nichrome within the strand.

IPSCs were evoked at 0.05 Hz by focal stimulation (posistioned 50-100 μm from the cell body) with a patch pipette containing 2 M NaCl. The stimulating electrode was repositioned until a reliable synaptic input to the cell was found. The orientation of the stimulating electrode, relative to the cell body, varied in the dorso-ventral and latero-medial axes. Stimulus intensity was between 2-30 V for EPSCs and IPSCs. Stimulus duration was 100 or 400 μs for both EPSCs and IPSCs. Monosynaptic EPSCs and IPSCs were identified by their ability to follow high frequency stimulation (10-20 Hz) with constant latency and the absence of failures (Hori *et al.*, 1996).

Paired-pulse stimulation can produce either facilitation or depression. Facilitation is thought to reflect an enhancement in neurotransmitter release due to the transient accumulation of Ca^{2+} close to release sites (Del Castillo & Katz, 1954; Zucker, 1989; Zucker & Regehr, 2002). Facilitation may also occur spuriously and may be due changes in both the number of synapses recruited and the probability of release at each site (Kim & Alger, 2001). By contrast, short-term paired-pulse depression is thought to reflect decreased transmitter release from the presynaptic terminal. This may involve depletion of transmitter stores, failure of the action potential to invade the presynaptic terminal, activation of presynaptic autoreceptors, or a reduction in activity-dependent I_{Ca} responsible for transmitter release (O'Donovan & Rinzel, 1997; Zucker & Regehr, 2002). Postsynaptic receptor desensitization (Mennerick & Zorumski, 1996; Trussell *et al.*, 1993) may also be involved. A postsynaptic effect of a neuromodulator is assumed to affect the first and second synaptic responses equally, whereas a presynaptic effect will affect the *ratio* of synaptic current amplitudes.

Paired EPSCs were generated by stimulating the dorsal root or dorsal root entry zone at a frequency of 0.05 Hz. The interval between paired stimuli ranged from 30-100 ms and 10-20 sweeps were averaged. Paired IPSCs were generated by focal stimulation at a frequency of 0.05 Hz. The interval between paired stimuli ranged from 50-100 ms and 10-20 sweeps were averaged. Paired-pulse data were expressed as the ratio of the amplitude of the second synaptic response relative to the first ($EPSC_2/EPSC_1$ or $IPSC_2/IPSC_1$). Paired-synaptic responses exhibited considerable inter-trial variability. For example, the first paired stimuli may have exhibited paired-pulse facilitation, but the next paired stimuli exhibited paired-pulse depression. When the data of 10-20 sweeps were averaged, the responses usually exhibited overall paired-pulse facilitation.

An npi SEC 05L amplifier was used for recording mEPSCs and mIPSCs. However, for some experiments, an Axopatch 1D (Axon Instruments, Burlingame, CA, USA) was used for recording mEPSCs and mIPSCs. In some experiments the secretagogue, ruthenium red (30 μM) was added to the aCSF. Ruthenium red is a polyvalent cation that blocks VDCCs and enhances miniature synaptic current frequency via a presynaptic and Ca^{2+}-independent mechanism (Hoffman & Lupica, 2000; Keller *et al.*, 2001; Sciancalepore *et al.*, 1998; Trudeau *et al.*, 1998). Data were only included in the analysis if the series resistance was below 25 MΩ and did not change by > 20% during the course of an experiment. Currents were filtered at 1 kHz (npi SEC 05L and Axopatch 1D), digitized at 5 kHz and data were stored on disk.

Histology

In some experiments, cells were filled with biocytin (0.1%) for *post-hoc* identification (Horikawa & Armstrong, 1988). This low concentration was chosen as biocytin has been reported to interfere with postsynaptic drug responses at higher concentrations (Eckert *et al.*, 2001). At the completion of recording, the patch pipette was slowly withdrawn from the cell and the slice was transferred to cold (4°C) 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 and stored overnight at 4°C.

Slices were rinsed 3 times with PBS and transferred to 20% sucrose in PBS. Slices were allowed to dehydrate for 2-5 d. Slices were sectioned on a cryostat at a thickness of 30-50 μm and transferred to individual wells in a six-well tissue culture dish (Costar, Cambridge, MA, USA) containing cold PBS. Sections were transferred to individual wells in a 24-well tissue culture dish (Corning, Corning, NY, USA), containing 0.3% Triton-X100 and streptavidin, Texas Red conjugate (Molecular Probes, Eugene, OR, USA), diluted to 1:50, and incubated for 50 min on a 3D rotator (Labline Instruments, Melrose Park, IL, USA). Sections were thoroughly rinsed with distilled water, transferred to slides, allowed to dry overnight and coverslipped with Cytoseal. A Leica DMRB epifluorescence microscope (Leica Canada, Willowdale, ON, Canada) was used to examine the tissue. The filter for Texas Red had an excitation band pass of 515-560 nm. Fluorescent images were viewed on a computer monitor coupled to a MTI 3 CCD video camera (Dage-MTI, Michigan City, IN, USA), digitized at a resolution of 300 dpi using Image-Pro 3.0 software (Media Cybernetics, Silver Spring, MD, USA) and saved to disk.

Data Analysis

Evoked EPSC, IPSC and K^+ current data were acquired and analyzed using pCLAMP 8.0 (Axon Instruments, Burlingame, CA, USA). Figures were produced with

Origin 5.0, 6.1 or 7.0 (OriginLab, Northampton, MA, USA) and Adobe Illustrator 10 (Adobe Software, San Jose, CA, USA). Statistical comparisons were made with paired t-tests, unless otherwise specified, using GraphPad InStat 3.05 (GraphPad Software, San Diego, CA, USA). Statistical significance was taken as $p < 0.05$ unless otherwise specified.

Analysis of Spontaneous and Miniature Postsynaptic Currents

Mini Analysis Program software (Synaptosoft, Decatur, GA, USA) was used to detect mEPSCs and mIPSCs. Mini Analysis detected events based on their amplitude and was set just above baseline noise levels.

The Mini Analysis Program used a variety of parameters for event detection. The sequence of event detection was as follows: the program took a specified portion of data to search for a local maximum value specified in μsec. After detecting a local maximum the program analyzed data points to the left of the local maximum by a value specified in μsec before the next peak and calculated an average baseline value. The peak amplitude was calculated by taking the amplitude at the local maximum and subtracting it from the average baseline value. The peak amplitude for the local maximum was compared to the user-defined amplitude threshold. If the peak amplitude was greater than or equal to the amplitude threshold the program proceeded to calculate the time-to-peak and decay time of the event. The time-to-peak of the event was calculated by finding the first data point to the left of the peak that was 0.5% of the peak amplitude. The time at this point was subtracted from the time at the peak of the event. The time-to-decay of the event was calculated by finding the first data point to the right of the peak. This value was represented as a fraction (0.37) of the peak amplitude and the difference between the time at this point and the time at the peak was calculated. The program also calculated the area under the curve by taking the integral of amplitude from the time-to-peak to the time-to-decay of the event. The area under the curve was compared to the area threshold and it was used to sort events that arose from noise. If the area under the curve was greater or equal to the area threshold, the program accepted the event and proceeded to the next peak on the data trace.

Spontaneous or miniature postsynaptic currents were detected automatically using an amplitude threshold of 10 pA and an area threshold of 15 fC. All detected events were then re-examined and visually accepted or rejected based on subjective visual examination. Mini Analysis Program was used to further analyze the data and to generate figures.

Cumulative frequency plots were generated to compare the effects of various drugs on the amplitude and inter-event interval distributions of mEPSCs and mIPSCs. Cumulative frequency plots ranked individual amplitudes or inter-event intervals in order of increasing size and plotted this rank value against the amplitude or inter-event interval size. The Kolmogorov-Smirnov two-sample test was used to compare control and drug distributions of amplitudes and inter-event intervals. The Kolmogorov-Smirnov tested the null hypothesis that two independent samples come from populations that are identical with respect to location and distribution. The Kolmogorov-Smirnov test was used because it compared the entire distribution of amplitudes or inter-event intervals rather than mean amplitude or mean inter-event intervals (Van der Kloot, 1991).

Distributions were considered statistically significant if $p < 0.05$. For each cell, typically 500 to 2000 events were analyzed.

Drugs and Chemicals

Neuropeptide Y Experiments

Neuropeptide Y was obtained from Tocris (Ballwin, MO, USA) or from Peptidec Technologies (Montreal, PQ, Canada). The Y1-agonist [Leu^{31}, Pro^{34}] NPY and the Y_2-agonist NPY 13-36 were from Tocris (Ballwin, MO, USA). The Y1-selective agonist [F^{7}, P^{34}] NPY and the Y2-selective agonist [ahx$^{5\text{-}24}$] NPY were kindly provided by Dr. Annette Beck-Sickinger (Institute for Biochemistry, University of Leipzig, Leipzig, Germany). The Y1-antagonist BIBP 3226 was from Peninsula Laboratories (San Carlos, CA, USA) and the Y2 antagonist BIIE0246 was a generous gift from Dr. Henri Doods, (Boehringer-Ingelheim, Biberach, Germany). Baclofen and ruthenium red were obtained from Tocris (Ballwin, MO, USA).

Morphine-3β-Glucuronide Experiments

[D-Ala^{2},N-Met-Phe^{4},Gly-ol^{5}]-enkephalin (DAMGO), naloxone and strychnine were obtained from Sigma (St. Louis, MO, USA). Nociceptin, bicuculline, CNQX and AP5 were from Tocris (Ballwin, MO, USA). QX-314 was supplied by AstraZeneca and TTX was from Alomone Labs (Jerusalem, Israel). Morphine sulfate was from British Drug Houses (Toronto, ON, Canada). Morphine-3β-glucuronide (M3G) was from Lipomed (Cambridge, MA, USA) and contained 0.28% morphine (HPLC analysis, Neurochemistry Research Unit, University of Alberta, Edmonton, Canada).

Figure 2-1. Schematic illustration of the dissected spinal cord. Spinal cord and muscle are removed *en bloc*, transferred to oxygenated, ice cold dissection solution and pinned to the bottom of the petri dish. The spinal cord is removed from the vertebral column, dura is removed and all ventral roots are cut at their exit zones. Dorsal roots are cut at their entry zone except for the L4-L5 roots.

Figure 2-1

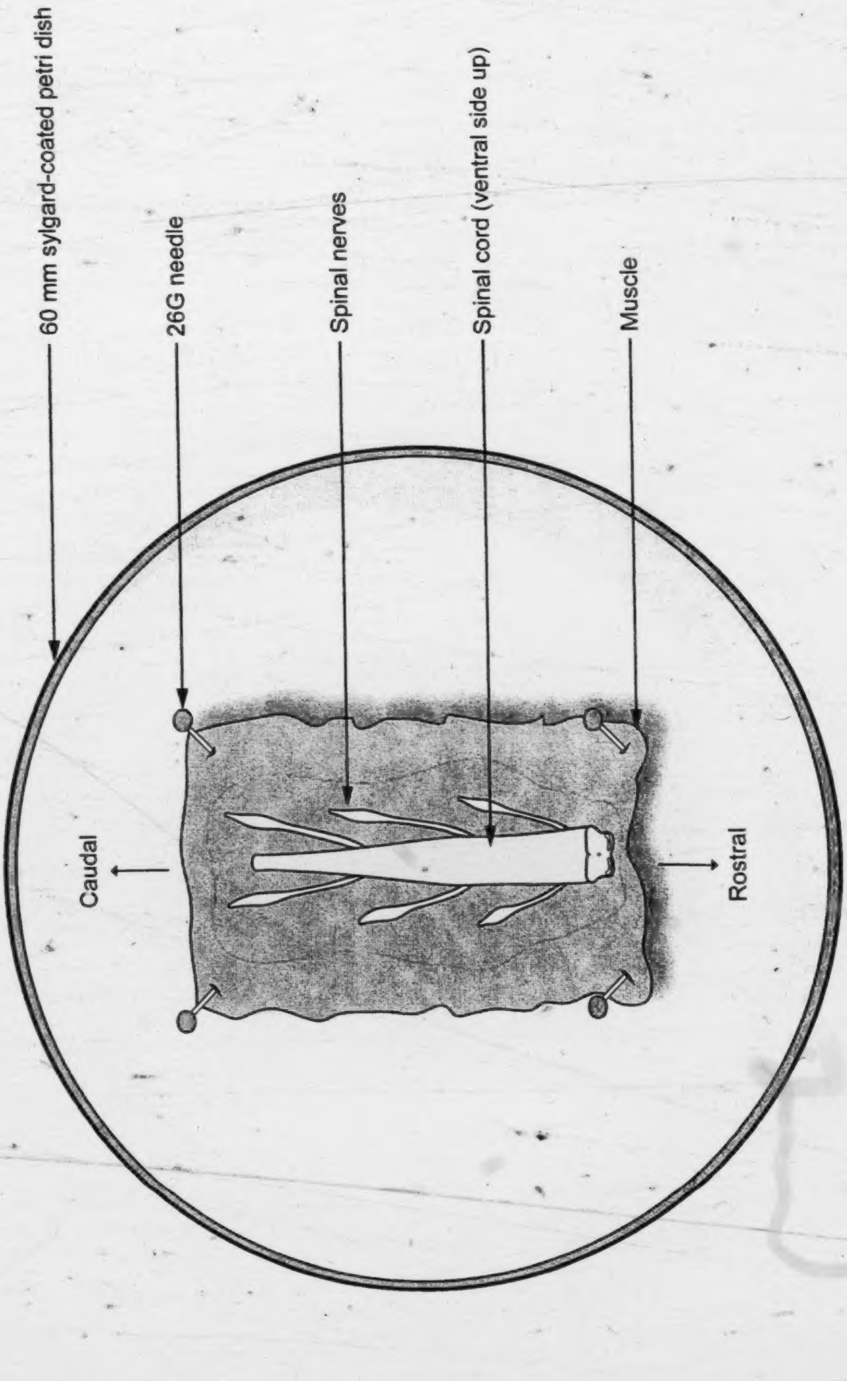

60 mm sylgard-coated petri dish
26G needle
Spinal nerves
Spinal cord (ventral side up)
Muscle
Caudal
Rostral

Figure 2-2. Diagram of the vibratome used for spinal cord slice preparation. The spinal cord is glued to a 4% agar block. The agar block is glued to the glass slide on the bottom of the petri dish and the spinal cord is submerged and bubbled with 95% O_2-5% CO_2 ice-cold dissection solution. The vibratome is advanced at ~1-4 mm/min and the advance speed is monitored continuously. Slices are typically cut at a thickness of 300-350 µm and five to six slices of lumbar spinal cord are routinely obtained.

Figure 2-2

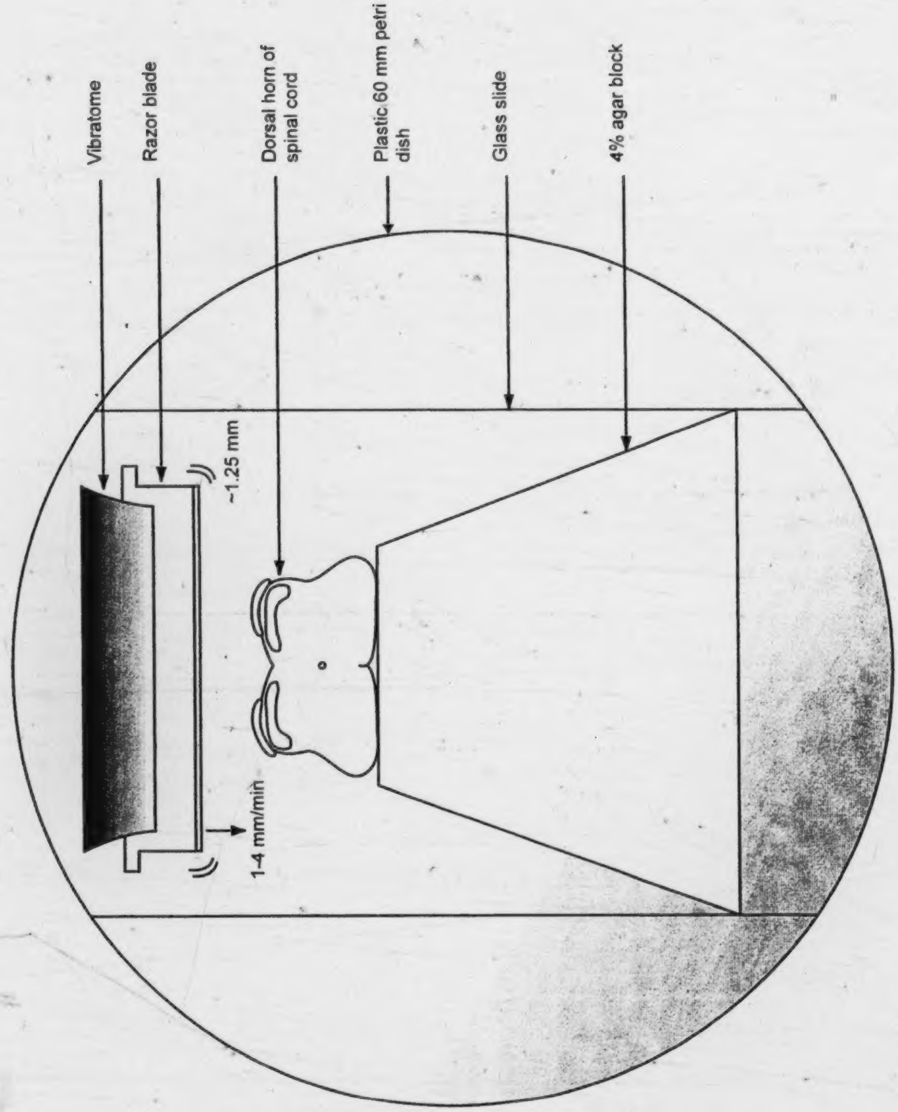

Vibratome
Razor blade
Dorsal horn of spinal cord
Plastic 60 mm petri dish
Glass slide
4% agar block
~1.25 mm
1-4 mm/min

Figure 2-3. Spinal cord slice storage chamber. Slices are transferred to the storage chamber, incubated for 1 h at 36°C and then stored at room temperature (~22°C) prior to recording. The chamber is made from a 150 mL glass beaker filled with dissection solution and bubbled with 95% O_2-5% CO_2. The slices are supported by nylon mesh covering the bottom of a plastic petri dish from which the bottom has been removed.

Figure 2-3

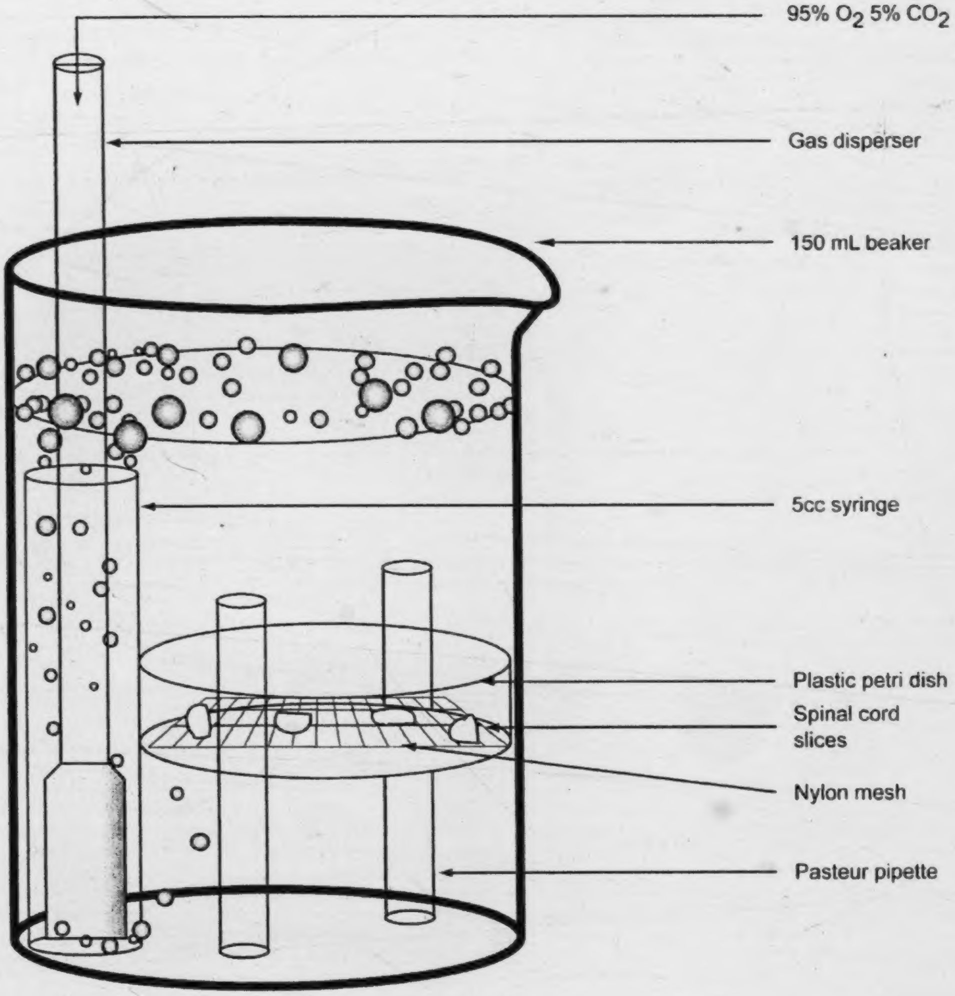

95% O_2 5% CO_2
Gas disperser
150 mL beaker
5cc syringe
Plastic petri dish
Spinal cord slices
Nylon mesh
Pasteur pipette

Figure 2-4. Optical setup for infrared-differential contrast videomicroscopy (IR-DIC). The substantia gelatinosa is illuminated with infrared (IR) light (λ = 750 nm) by inserting an IR filter in the light path before the polarizer. Substantia gelatinosa neurons are viewed with a Zeiss 40X 0.75NA water-immersion lens and conventional DIC optics. The image is magnified another 4X, detected with an IR-sensitive camera and displayed on a video monitor.

Figure 2-4

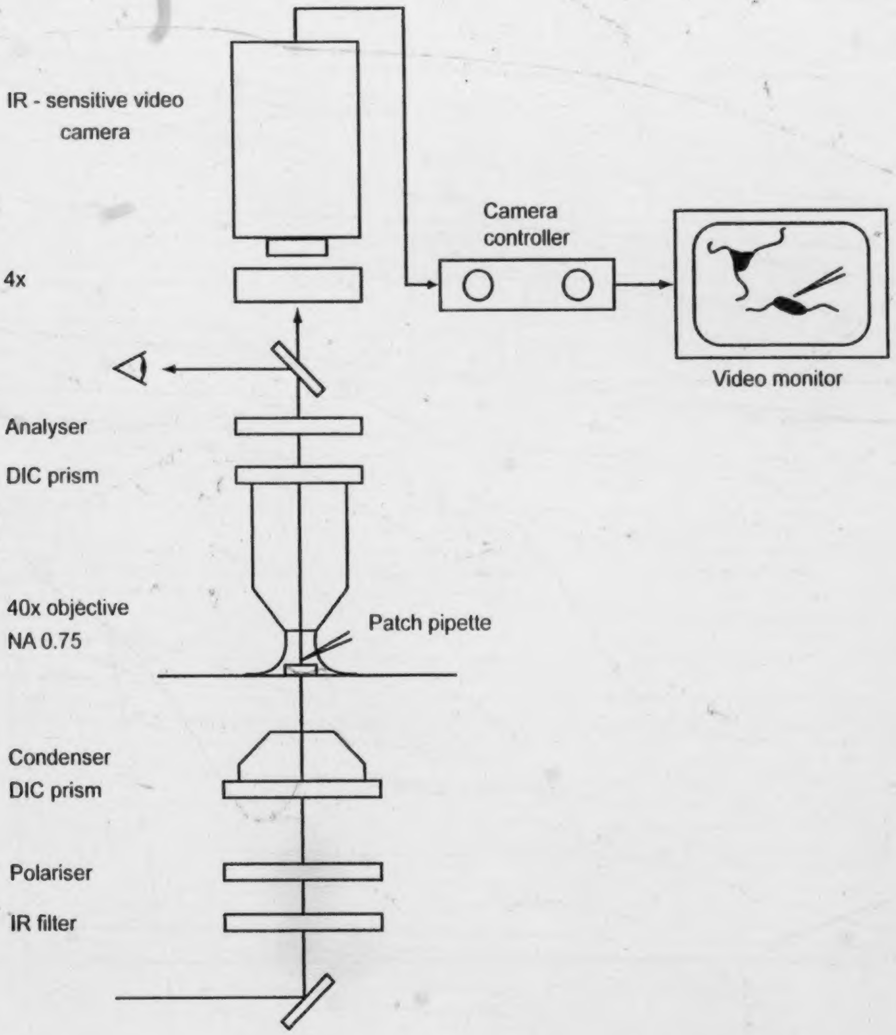
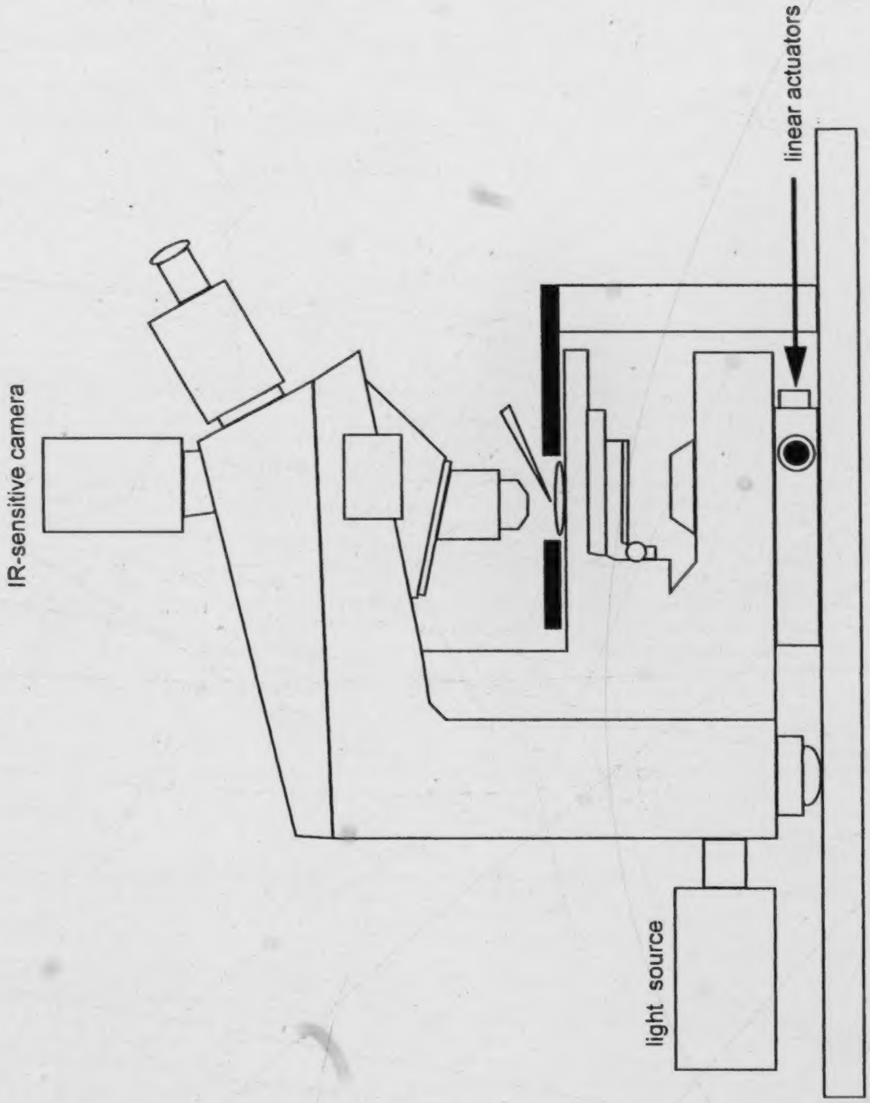

IR - sensitive video camera
Camera controller
4x
Video monitor
Analyser
DIC prism
40x objective NA 0.75
Patch pipette
Condenser DIC prism
Polariser
IR filter

Figure 2-5. Diagram of the Zeiss Axioskop FS used for visualizing substantia gelatinosa neurons. The microscope is mounted on two linear actuators, allowing the microscope to be moved in the *x*- and *y*-axes, without affecting the relative position of the recording and stimulating electrodes. The recording chamber is mounted on a separate fixed stage.

Figure 2-5


IR-sensitive camera
linear actuators
light source

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Chapter 3

Electrophysiological and Pharmacological Properties of Substantia Gelatinosa Neurons in the Spinal Cord Slice

Introduction

This chapter is mainly descriptive and illustrates some of the fundamental synaptic and pharmacological properties of substantia gelatinosa neurons. These experiments were performed with the use of IR-DIC videomicroscopy and recordings were made from visually-identified neurons. These experiments sought to determine if the spinal cord slices would exhibit similar synaptic and pharmacological properties described by other research laboratories (Grudt & Henderson, 1998; Lai *et al.*, 1997; Yoshimura & Jessell, 1989b; Yoshimura & North, 1983). This chapter also includes a brief morphological description of some of the cell types in the substantia gelatinosa.

The *in vitro* adult rat spinal cord slice preparation was first described by Yoshimura and North (North & Yoshimura, 1984; Yoshimura & North, 1983) and this preparation has provided a means to study the cellular properties of substantia gelatinosa neurons (lamina I and II). In rat substantia gelatinosa the majority of primary afferent input is from Aδ and C-fibres (Yoshimura & Jessell, 1989b). These fibres release glutamate (Schneider & Perl, 1988; Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1993), which activates AMPA, NMDA and kainate receptors (Bardoni *et al.*, 1998; Kerchner *et al.*, 2001b; Kerchner *et al.*, 2001a; Li *et al.*, 1999; Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1992; Yoshimura & Nishi, 1993). Although direct immunocytochemical evidence is lacking for excitatory interneurons in lamina II (Todd & Spike, 1993), calbindin-D28K (Antal *et al.*, 1991; Polgar & Antal, 1995) and GluR2/3 subunits (Kerr *et al.*, 1998) are thought to be markers for excitatory interneurons in the dorsal horn. It is likely that stalked cells and possibly some small islet cells contain glutamate (Spike & Todd, 1992).

GABA and glycine are the principal inhibitory neurotransmitters in the substantia gelatinosa (Malcangio & Bowery, 1996; Todd & Spike, 1993). GABA-IR neurons comprise about 30% of the total population of neurons in lamina II (Magoul *et al.*, 1987; Todd & McKenzie, 1989; Todd & Sullivan, 1990) and most GABA immunoreactivity at lamina II synapses likely originates from local inhibitory interneurons (Todd, 1996). $GABA_A$ receptor binding sites are present on fine diameter, capsaicin-sensitive primary afferent fibres (Coggeshall & Carlton, 1997; Singer & Placheta, 1980). GABA-IR cells are typically described as 'large' islet cells, whereas 'small' islet cells are not GABA-IR (Spike & Todd, 1992; Todd & McKenzie, 1989).

Aδ-fibre stimulation generates EPSCs that are often followed by a short or long duration, bicuculline- and strychnine-sensitive IPSC (Yoshimura & Nishi, 1993; Yoshimura & Nishi, 1995). Focal stimulation generates monosynaptic GABA- and glycine-mediated IPSCs that arise from the activation of local inhibitory interneurons (Chery & de Koninck, 1999; Grudt & Henderson, 1998; Keller *et al.*, 2001; Yoshimura & Nishi, 1995). Similarly, in trigeminal nucleus pars caudalis evoked IPSCs and TTX-insensitive miniature IPSCs are blocked by bicuculline and strychnine (Grudt & Henderson, 1998).

$GABA_B$ receptors are present on the terminals of primary afferent fibres and local interneurons where they regulate transmitter release (Ataka *et al.*, 2000; Chery & de Koninck, 2000; Grudt & Henderson, 1998; Iyadomi *et al.*, 2000; Malcangio & Bowery, 1993; Malcangio & Bowery, 1996). $GABA_B$ receptors are also expressed postsynaptically and hyperpolarize dorsal horn neurons by activating a K^+ conductance (Kangrga *et al.*, 1991).

Glycine immunoreactivity is present in lamina I and II and is typically co-localized in GABA-IR cell bodies (Todd, 1990; Todd, 1996; Todd & Sullivan, 1990) and cells immunoreactive for both GABA and glycine are indistinguishable from neurons that are only GABA-IR (Spike & Todd, 1992; Todd & Sullivan, 1990). GABA and glycine receptors are often co-localized at synapses in the dorsal horn (Todd *et al.*, 1996) and GABA and glycine are co-released from individual vesicles at some synapses (Chery & de Koninck, 1999; Keller *et al.*, 2001).

Recently, Grudt & Perl (Grudt & Perl, 2002) completed a detailed analysis of the morphology, membrane properties and synaptic inputs for hamster lamina I and II cells. They subdivided lamina II neurons into five morphological categories: islet, central, medial—lateral, radial and vertical. The characteristics of these cells have been described in detail in the General Introduction.

Methods

The methods for recording from substantia gelatinosa neurons were identical to those described in Chapter 2. In some experiments, a K^+-gluconate-based internal solution was used. When using this solution, the holding potential was –60 or –70 mV for recording EPSCs and 0 mV for recording IPSCs. When using a CsCl-based internal solution, the holding potential was –60 or –70 mV for recording evoked EPSCs and

IPSCs. A CsCl-based internal for recording mEPSCs and mIPSCs and cells were voltage-clamped at –70 mV.

Results

Evoked Excitatory Postsynaptic Currents in Substantia Gelatinosa Neurons

Whole-cell patch-clamp recordings were obtained from substantia gelatinosa neurons from slices maintained *in vitro* for up to 10 h and stable recordings were made from individual neurons for up to 3 h. Cells had an average input resistance of 392 ± 14 MΩ (n = 119) and cell capacitance of 45 ± 1 pF (n = 119) with a K^+-gluconate internal solution in the recording electrode.

In the presence of bicuculline (10 μM) and strychnine (1 μM), stimulation of the dorsal root or dorsal root entry zone generated EPSCs in substantia gelatinosa neurons at a holding potential of –60 or –70 mV. Superfusion of the AMPA receptor antagonist, CNQX (10 μM), and the NMDA receptor antagonist, AP5 (50 μM), abolished the evoked EPSC. Sample current traces of the effect of AP5 and CNQX on the evoked EPSC are illustrated in Figure 3-1A. The time course for the effects of these antagonists is shown in Figure 3-1B. In this cell, the effect of AP5 and CNQX took about 5 min to develop and only partially recovered over a washout period of about 25 min.

Evoked Inhibitory Postsynaptic Currents in Substantia Gelatinosa Neurons

Focal stimulation in the presence of strychnine (1 μM), AP5 (50 μM) and CNQX (10 μM) elicited IPSCs in substantia gelatinosa neurons. The IPSC was completely blocked by bicuculline (10 μM), suggesting it was mediated by $GABA_A$ receptors. Averaged current traces (n = 6) illustrating the effect of bicuculline on the IPSC is shown in Figure 3-2A. The time course for the effects of bicuculline is shown in Figure 3-2B. In this cell, the response to bicuculline took about 5 min to develop and recovered slightly over a period of about 55 min.

In the presence of bicuculline (10 μM), AP5 (50 μM) and CNQX (10 μM) focal stimulation elicited IPSCs in substantia gelatinosa neurons. This IPSC was completely blocked by strychnine (1 μM), suggesting it was mediated by glycine receptors. Averaged current traces (n = 6) illustrating the effect of bicuculline on the IPSC is shown in Figure 3-2C. The time course for the effects of glycine is shown in Figure 3-2D. In this cell, the response to bicuculline took about 5 min to develop and only recovered slightly over a period of about 20 min. Note that the decay time for the glycinergic IPSC shown in Figure 3.2C is faster that for the $GABA_A$-mediated IPSC shown in Figure 3.2A. A detailed kinetic analysis of the $GABA_A$- and glycine-mediated IPSCs was not carried out.

Detection of Monosynaptic Excitatory and Inhibitory Postsynaptic Currents

To facilitate interpretation of drug responses, only monosynaptic EPSCs and IPSCs were selected for further analysis. The EPSCs and IPSCs showed graded responses to increasing stimulus intensity, suggesting discrete fibres were not recruited. Monosynaptic responses were identified by two criteria: (1) their ability to follow high frequency stimulation (10 Hz) with reduced amplitude, but without a change in response latency; and (2) the response latency did not change with increasing stimulus intensity

(Hori *et al.*, 1996; Li & Zhuo, 1998). These criteria were applied at the conclusion of all experiments and polysynaptic currents (i.e. variable response latency and amplitude) were excluded from further analysis. An example of a monosynaptic EPSC is illustrated in Figure 3-3A. EPSCs were generated by stimulating the dorsal root or dorsal root entry zone at 10 Hz. In Figure 3-3B, an example of a monosynaptic IPSC is shown. IPSCs were generated by focal stimulation at 10 Hz.

Baclofen Suppresses Evoked Excitatory and Inhibitory Postsynaptic Currents

The $GABA_B$ agonist, baclofen, suppresses excitatory and inhibitory synaptic transmission in the substantia gelatinosa (Ataka *et al.*, 2000; Chery & de Koninck, 2000; Grudt & Henderson, 1998; Iyadomi *et al.*, 2000; Malcangio & Bowery, 1993; Malcangio & Bowery, 1996). Baclofen was used to determine if the methods used to obtain spinal cord slices allowed neurons to retain reliable and robust G-protein coupled responses.

In the presence of bicuculline (10 μM) and strychnine (1 μM), stimulation of the dorsal root or dorsal root entry zone generated EPSCs in substantia gelatinosa neurons at a holding potential of –60 or –70 mV. Superfusion of baclofen (15-30 μM) caused robust suppression of the EPSC (57.0 ± 10.3%, n = 4). Raw data traces illustrating the effect of baclofen (30 μM) on the evoked EPSC are shown in Figure 3-4A and the time course of action for baclofen is illustrated in Figure 3-4B. In this particular cell, the effect of baclofen took about 3 min to develop and only partially recovered over a period of about 25 min.

In the presence of AP5 (50 μM) and CNQX (10 μM), focal stimulation generated IPSCs at a holding potential of 0 mV (K^+-gluconate internal) or -70 mV (CsCl-based internal). Superfusion of baclofen (15-30 μM) caused robust suppression of the evoked IPSC (65.2. ± 4.31%, n = 5). Raw data traces illustrating the effect of baclofen (15 μM) on the evoked IPSC are shown in Figure 3-4C and the time course of action for baclofen is illustrated in Figure 3-4D. In this particular cell, the effect of baclofen took about 3 min to develop and completely recovered after about 15 min of drug washout.

Morphology of Substantia Gelatinosa Neurons

Most anatomical descriptions of lamina II neurons have relied on parasagittal or sagittal spinal cord sections (Bennett *et al.*, 1980; Gobel, 1978; Grudt & Perl, 2002; Schneider & Perl, 1988; Spike & Todd, 1992) in which their dendritic aborizations can be easily visualized. Because transverse slices were used in the present experiments, limited conclusions can be made regarding cell morphology. However, based on cell body shape, orientation of their dendrites and location within lamina II, most recordings were obtained from neurons which could be broadly categorized as islet cells (n = 34) (Aimar *et al.*, 1998; Light *et al.*, 1979; personal communication, A.B. MacDermott, Columbia University).

Electrophysiological recordings and photomicrographs of two lamina II neurons are shown in Figure 3-5. In Figure 3-5A, raw traces illustrating the effect of baclofen (15 μM) on the evoked IPSC are shown. NPY (300 nM) was also tested on this cell but had no effect. Figure 3-5B is a photomicrograph of the neuron described in Figure 3-5A. Based on cell body shape and dendritic aborizations, this neuron is possibly an islet cell. The majority of biocytin-filled cells had morphology similar to this neuron. Figure 3-5C shows raw data traces of mEPSCs recorded from another substantia gelatinosa neuron.

Figure 3-5D is a photomicrograph of the neuron described in Figure 3-5C. This cell had a pyramidal-shaped cell body, was located in the inner portions of lamina II and resembled neurons described by (Woolf & Fitzgerald, 1983). The dendrites of this cell extended into deeper laminae suggesting it may be a stalked cell (Gobel, 1978).

Discussion

These experiments verified the procedures used for spinal cord slice preparation. These slices had similar pharmacological and synaptic properties to previous studies (Yoshimura & Jessell, 1989a; Yoshimura & Jessell, 1989b; Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1992; Yoshimura & Nishi, 1993; Yoshimura & Nishi, 1995; Yoshimura & North, 1983). These experiments confirmed that excitatory synaptic events were mediated by AMPA and NMDA receptors and inhibitory synaptic transmission was mediated by $GABA_A$ and glycine receptors. The application of high frequency stimulus protocols was used to determine if these synaptic responses were mono- or polysynaptic. They also demonstrated G-protein-coupled responses were intact in presynaptic neurons of substantia gelatinosa neurons, as application of the $GABA_B$ agonist, baclofen, suppressed excitatory and inhibitory synaptic transmission. Lastly, *post-hoc* morphological identification of biocytin-filled cells indicated that most electrophysiological recordings were obtained from islet cells.

Evoked EPSCs were completely blocked by the AMPA receptor antagonist, CNQX, and the NMDA receptor antagonist, AP5. This finding concurs with previous studies showing that fast excitatory synaptic transmission in the substantia gelatinosa is mediated primarily by AMPA and NMDA receptors (Bardoni *et al.*, 1998; Schneider &

Perl, 1988; Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1992). Recent studies show that EPSCs activated by stimulating high-threshold primary afferents are mediated in part by kainite receptors (Kerchner *et al.*, 2002; Li *et al.*, 1999). These receptors are also present on terminals of primary afferent fibres and interneurons in the dorsal horn where they regulate the release of glutamate, GABA and glycine (Kerchner *et al.*, 2001a; Kerchner *et al.*, 2001b; Kerchner *et al.*, 2002). However, these experiments do not assess the relative contribution of kainite receptors to excitatory synaptic transmission or its modulatory role in the dorsal horn.

Evoked IPSCs were bicuculline- and strychnine-sensitive, suggesting that the IPSC was mediated by both $GABA_A$ and glycine receptors and these findings agree with previous research (Chery & de Koninck, 1999; Chery & de Koninck, 2000; Grudt & Henderson, 1998; Keller *et al.*, 2001; Yoshimura & Nishi, 1995). Although a detailed analysis was not performed, the decay time of the bicuculline-sensitive IPSC was consistently slower than the strychnine-sensitive IPSC and is similar to previous findings (Chery & de Koninck, 1999; Chery & de Koninck, 2000; Grudt & Henderson, 1998; Keller *et al.*, 2001).

Almost all evoked EPSCs and IPSCs were monosynaptic. Polysynaptic IPSCs were observed infrequently and were not included in any data analysis. Monosynaptic responses were selected for study as their constant latency and amplitude allowed for consistent interpretation of drug responses. Because polysynaptic events vary in both amplitude and latency (Hori *et al.*, 1996), they are unsuitable for interpreting the actions of neuromodulators on synaptic transmission.

The GABA_B agonist, baclofen, potently inhibited evoked EPSCs and IPSCs. This observation is consistent with previous reports that baclofen suppresses excitatory and inhibitory synaptic transmission in the dorsal horn, primarily by activating presynaptic GABA_B receptors (Ataka *et al.*, 2000; Chery & de Koninck, 2000; Grudt & Henderson, 1998). It also confirms that the procedures used for spinal cord slice preparation did not compromise G-protein coupled responses, at least in presynaptic terminals.

Two recent studies (Grudt & Perl, 2002; Prescott & de Koninck, 2002) have correlated cell morphology with the action potential firing properties, ionic currents and afferent inputs to lamina I and II neurons. In these studies at least four distinct neuronal types are present in rat lamina I (Prescott & de Koninck, 2002) and at least five different cell types are found in hamster lamina II (Grudt & Perl, 2002). In this study most electrophysiological recordings were likely obtained from neurons that could be broadly categorized as islet cells. This is supported cell input resistance which is very similar to the values reported for islet cells by Grudt & Perl (2002). Because the dendrites of lamina II neurons extend primarily along the rostro-caudal axis of the spinal cord (Gobel, 1978; Spike & Todd, 1992), limited conclusions regarding their morphology could be made using a transverse slice preparation.

In summary, these experiments validated the procedures used to prepare spinal cord slices for IR-DIC videomicroscopy in conjunction with patch-clamp electrophysiology. They demonstrated that EPSCs were mediated via AMPA and NMDA receptors, IPSCs were mediated via GABA_A and glycine receptors and the majority of these currents were monosynaptic. Responses to the GABA_B agonist, baclofen, confirmed GPCRs were intact and capable of modulating synaptic activity.

Lastly, biocytin-labeling suggested that electrophysiological recordings were obtained primarily from islet cells.

Figure 3-1. Primary afferent stimulation generates AMPA- and NMDA receptor-mediated EPSCs in substantia gelatinosa neurons. EPSCs were evoked by stimulating the dorsal root in the presence of 10 μM bicuculline and 1 μM strychnine. A, averaged traces (n = 6) of evoked EPSCs before during and after application of the AMPA antagonist, CNQX (10 μM), and the NMDA antagonist, AP5 (50 μM). Note the EPSC is abolished in the presence of CNQX and AP5. B, time course of the changes in amplitude of evoked EPSCs during application of CNQX and AP5. The neuron was voltage-clamped at a holding potential of –70 mV with a CsCl-based internal solution in the recording electrode.

Figure 3-1

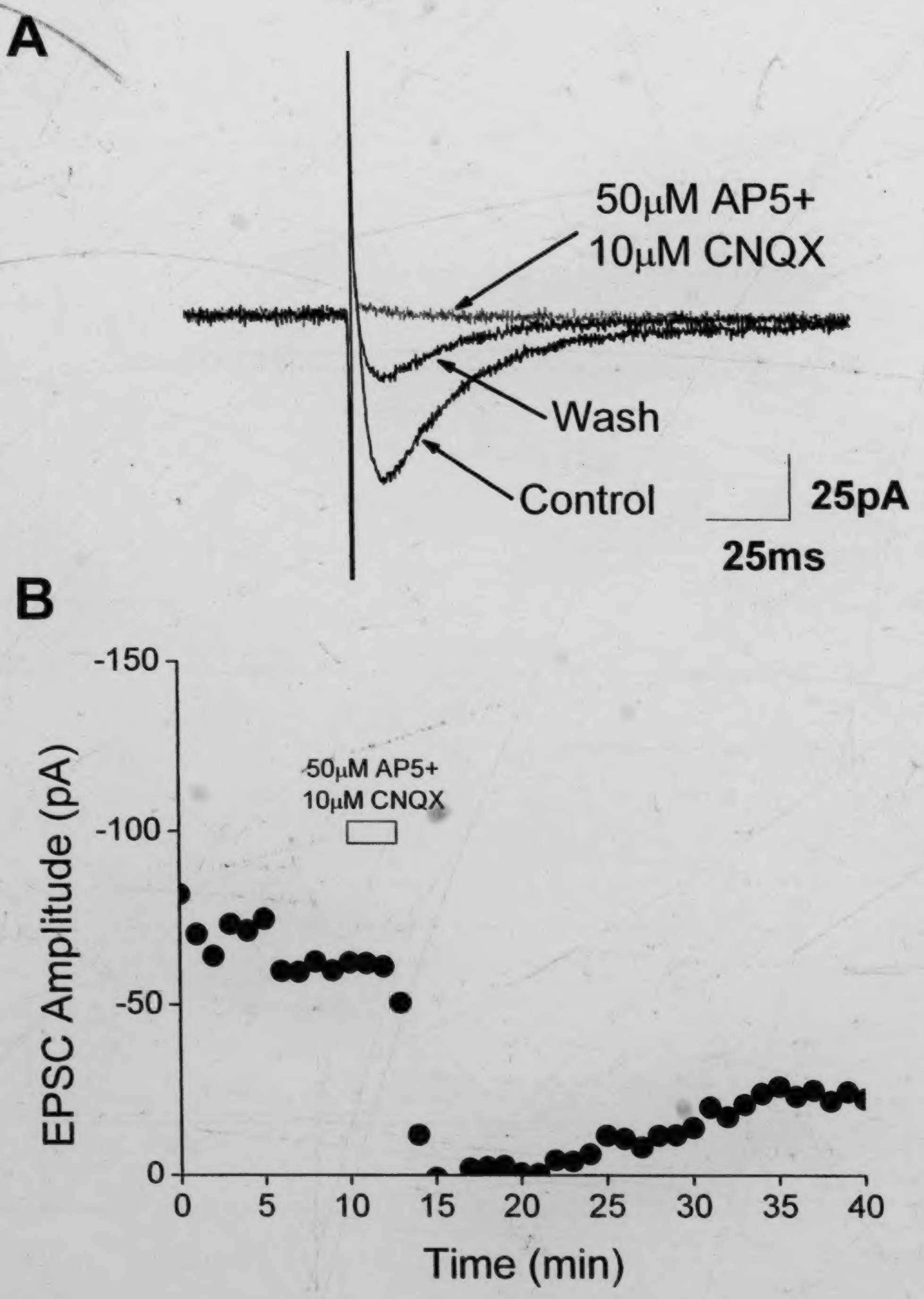

A
50μM AP5+
10μM CNQX
Wash
Control
25pA
25ms
B
-150
-100
-50
0
EPSC Amplitude (pA)
50μM AP5+
10μM CNQX
0
5
10
15
20
25
30
35
40
Time (min)

Figure 3-2. Focal stimulation generates GABA- and glycine-mediated IPSCs in substantia gelatinosa neurons. GABA-mediated IPSCS were evoked by focal stimulation in the presence of AP5 (50 μM), CNQX (10 μM) and strychnine (1 μM). A, averaged traces (n = 6) of evoked IPSCs before, during and after application of the $GABA_A$ antagonist, bicuculline (10 μM). Note the IPSC is abolished after bicuculline superfusion. B, time course of the changes in amplitude of evoked IPSCs during application of bicuculline. The neuron was voltage-clamped at a holding potential of -70 mV. Glycine-mediated IPSCs were evoked by focal stimulation in the presence of AP5 (50 μM), CNQX (10 μM) and bicuculline (10 μM). C, averaged traces (n = 6) of evoked IPSCs before, during and after application of the glycine receptor antagonist, strychnine (1 μM). Note the IPSC is abolished after strychnine superfusion. D, time course of the changes in amplitude of evoked IPSCs during application of strychnine. The neuron was voltage-clamped at a holding potential of –70 mV with a CsCl-based internal solution in the patch pipette. Note the differences in time scale for A and C.

Figure 3-2

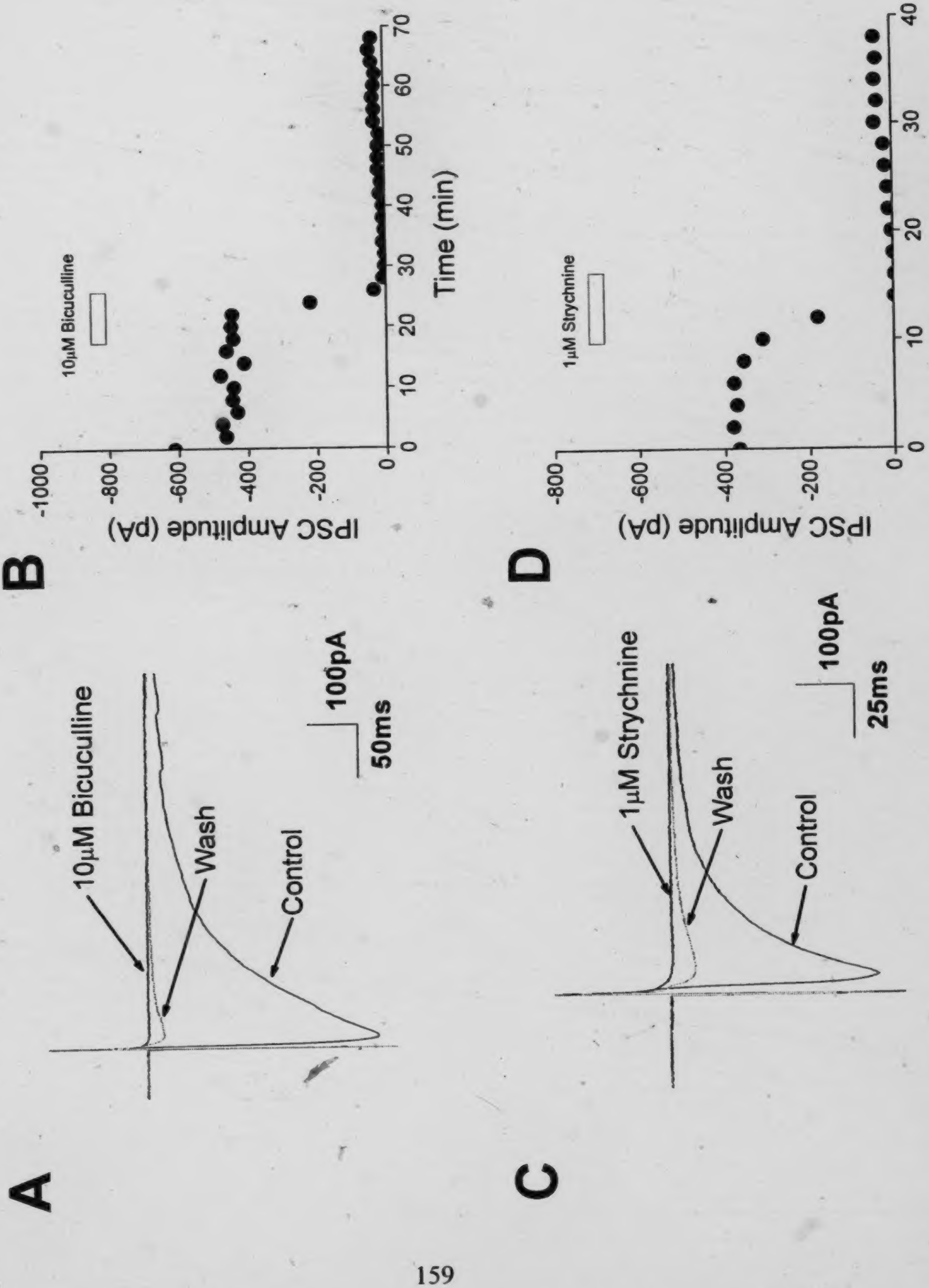
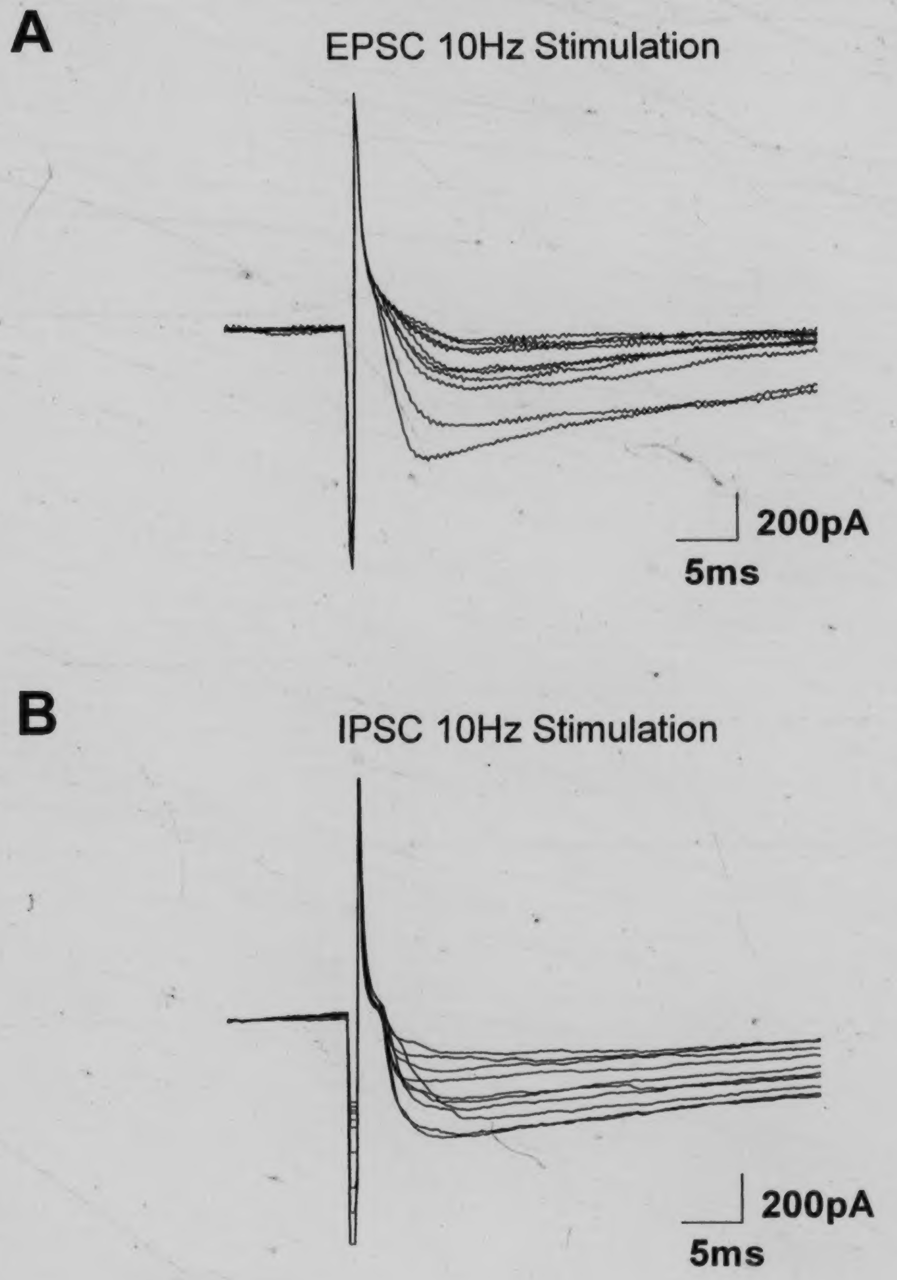

A
10µM Bicuculline
Wash
Control
100pA
50ms
B
IPSC Amplitude (pA)
-1000
-800
-600
-400
-200
0
10µM Bicuculline
0
10
20
30
40
50
60
70
Time (min)
C
1µM Strychnine
Wash
Control
100pA
25ms
D
IPSC Amplitude (pA)
-800
-600
-400
-200
0
1µM Strychnine
0
10
20
30
40
Time (min)

Figure 3-3. High frequency stimulation evokes monosynaptic EPSCs and IPSCs in substantia gelatinosa neurons. EPSCs were evoked by stimulating the dorsal root at 10 Hz in the presence of 10 μM bicuculline and 1 μM strychnine. A, EPSCs exhibit constant latency, but decreased amplitude with each successive sweep. The neuron was voltage-clamped at a holding potential of –70 mV with a CsCl-based internal solution in the patch pipette. GABA- and glycine-mediated IPSCs were evoked by focal stimulation at 10 Hz in the presence of AP5 (50 μM) and CNQX (10 μM). B, IPSCs exhibit constant latency, but decreased amplitude with each successive sweep. The neuron was voltage-clamped at a holding potential of –70 mV with a CsCl-based internal solution in the patch pipette.

Figure 3-3



A

EPSC 10Hz Stimulation

B

IPSC 10Hz Stimulation

Figure 3-4. GABA$_B$ receptor activation modulates excitatory and inhibitory synaptic responses in substantia gelatinosa neurons. EPSCs were evoked by stimulating the dorsal root in the presence of 10 μM bicuculline and 1 μM strychnine. A, averaged traces (n = 3) of EPSCs before, during and after application of 30 μM baclofen. Note baclofen (30 μM) almost completely abolishes the EPSC. EPSCs were evoked from a holding potential of –70 mV with a CsCl-based internal solution in the patch pipette. B, time course of the changes in amplitude of evoked EPSCs during application of baclofen. IPSCs were evoked by focal stimulation in the presence of 10 μM CNQX and 50 μM AP5. C, averaged traces (n = 3) of IPSCs evoked before, during and after application of 15 μM baclofen. Note baclofen strongly inhibits the evoked IPSC. The neuron was voltage-clamped at a holding potential of 0 mV with a K$^+$-gluconate-based internal solution in the patch pipette. D, time course of the changes in amplitude of evoked IPSCs during application of baclofen.

Figure 3-4

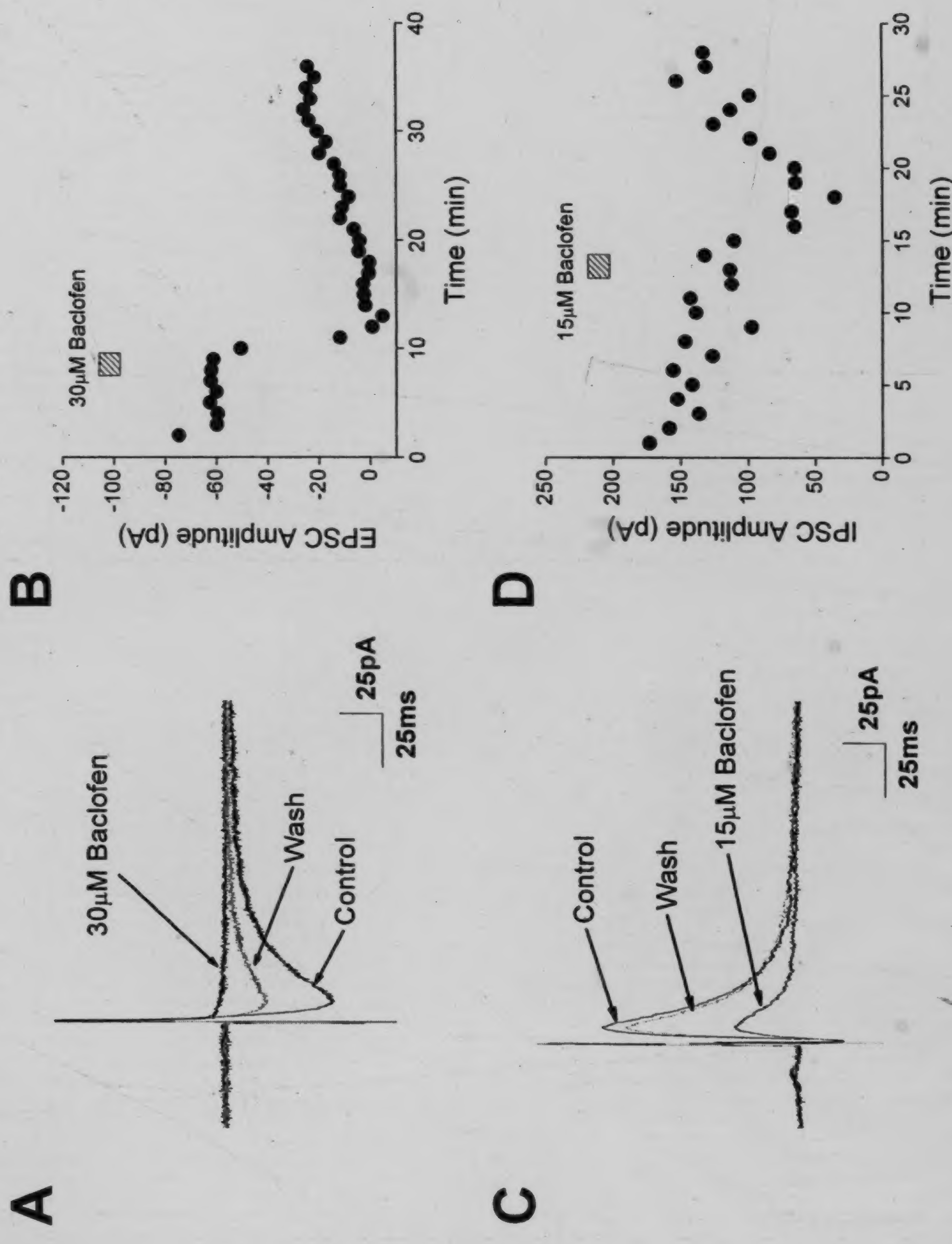
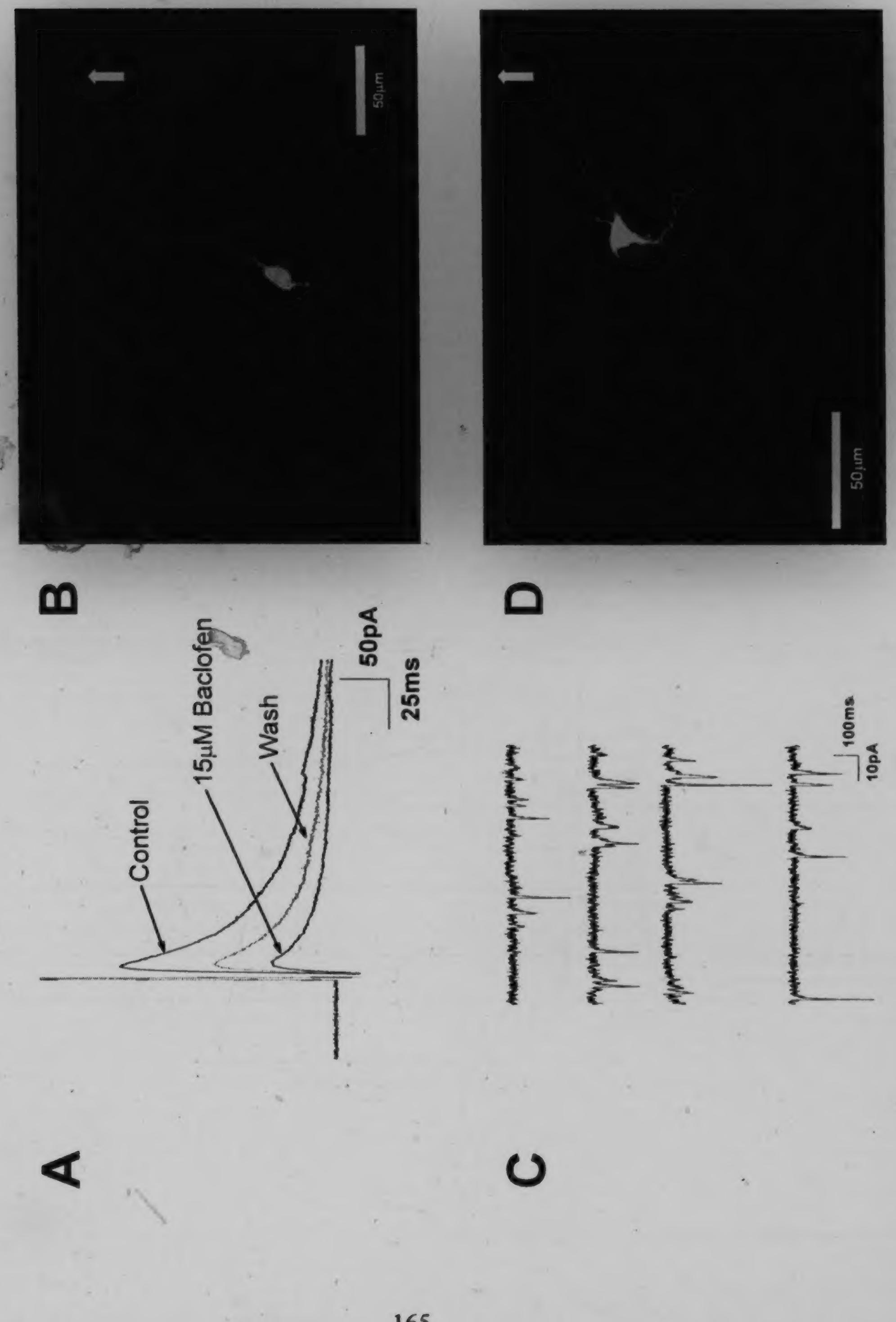

A
30µM Baclofen
Wash
Control
25pA
25ms
B
30µM Baclofen
EPSC Amplitude (pA)
-120
-100
-80
-60
-40
-20
0
0
10
20
30
40
Time (min)
C
Control
Wash
15µM Baclofen
25pA
25ms
D
15µM Baclofen
IPSC Amplitude (pA)
250
200
150
100
50
0
0
5
10
15
20
25
30
Time (min)

Figure 3-5. Patch-clamp recordings and photomicrographs obtained from biocytin-filled rat spinal cord substantia gelatinosa neurons. A, averaged traces (n = 6) of IPSCs evoked by focal stimulation in the presence of 10 μM CNQX and 50 μM AP5 before during and after application of 15 μM baclofen. Note baclofen potently suppresses the IPSC. IPSCs were evoked from a holding potential of 0 mV with a K^+-gluconate-based internal solution in the patch pipette. B, photomicrograph of the cell described in A. The image was captured from 50 μm thick transverse sections. The cell was visualized and photographed through a bandpass filter appropriate for Texas Red. Note image has been converted to grayscale for clarity. Dorsal is shown up. See text for additional details. C, raw data traces of mEPSCs, recorded in the presence of TTX (1 μM), from another substantia gelatinosa neuron. This cell was voltage-clamped at a holding potential of –70 mV with a CsCl-based internal solution in the patch pipette D, photomicrograph of the neuron described in C. The image was captured from 50 μm transverse sections. The cell was visualized and photographed through a bandpass filter appropriate for Texas Red. Note image has been converted to grayscale for clarity. Arrows indicate dorsal surface of spinal cord. See text for additional details.

Figure 3-5


A
Control
15μM Baclofen
Wash
50pA
25ms
B
50μm
C
100ms
10pA
D
50μm

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Chapter 4

Pre- and Postsynaptic Actions of Neuropeptide Y on Substantia Gelatinosa Neurons

Introduction

As described in the General Introduction, neuropathic pain often responds poorly to opioid treatment. This observation is supported by decreased μ-opioid receptor expression on dorsal root ganglia (DRG) neurons and decreased immunoreactivity for μ- and δ-opioid receptors in the spinal dorsal horn following peripheral nerve axotomy (de Groot *et al.*, 1999; Zhang *et al.*, 1998b; Zhang *et al.*, 1998a). Furthermore, the effect of morphine on N-type Ca^{2+} current in DRG cell bodies is considerably reduced in axotomized animals (Abdulla & Smith, 1998). Thus, non-opioid, spinal peptidergic mechanisms represent a potential target for the treatment of neuropathic pain.

By contrast, levels of NPY expression increase in the dorsal root ganglia and dorsal horn following axotomy or a chronic constriction injury (CCI) (Munglani *et al.*, 1996; Villar *et al.*, 1989; Wakisaka *et al.*, 1991). NPY Y2 receptor levels also increase in DRG (Mantyh *et al.*, 1994; Zhang *et al.*, 1994b) where they couple to N-type Ca^{2+} channels (Abdulla & Smith, 1999). In addition, NPY mRNA and peptide levels increase in spinal cord lamina II after axotomy. Because NPY acts as an analgesic when applied intrathecally, it represents an attractive target for the treatment of neuropathic pain (Hua *et al.*, 1991; Xu *et al.*, 1994). Furthermore, many of the cellular actions of opioids, which involve suppression of calcium current, decrease in transmitter release and activation of GIRK currents (Grudt & Henderson, 1998; Grudt & Williams, 1993; Grudt & Williams, 1994; Hori *et al.*, 1992; Yoshimura & North, 1983) are shared by NPY (Bleakman *et al.*, 1991; Colmers *et al.*, 1991; Colmers & Bleakman, 1994; Qian *et al.*, 1997; Toth *et al.*, 1993; Zidichouski *et al.*, 1990). Therefore, the effects of NPY and NPY receptor-selective ligands were tested on dorsal horn neurons of adult rats. Actions of NPY were also compared to those of the μ-agonist, DAMGO.

Methods

The methods for recording from substantia gelatinosa neurons were identical to those described in Chapter 2, except the slices were incubated at 36°C for 1h prior to recording and then stored at room temperature. In some experiments, a K^+-gluconate based internal solution was used. When using this solution, the holding potential was –60 or –70 mV for recording EPSCs and 0 mV for recording IPSCs. When using a CsCl-based internal solution the holding potential was –60 or –70 mV for recording evoked EPSCs and IPSCs. For recording mEPSCs and mIPSCs, a CsCl-based internal was used and cells were held at –60 or –70 mV.

Results

NPY and NPY-selective Agonists: Excitatory Synaptic Transmission

Evoked Excitatory Postsynaptic Currents

Whole-cell patch-clamp recordings were obtained from substantia gelatinosa neurons from slices maintained *in vitro* for up to 10h and stable recordings were made from individual neurons for up to 3h.

In the presence of bicuculline (10 μM) and strychnine (1 μM), stimulation of the dorsal root or dorsal root entry zone generated EPSCs in substantia gelatinosa neurons at a holding potential of –70 mV. Superfusion of NPY (300 nM or 1 μM) reduced EPSC amplitude by an average of 45.5 ± 4.64% in 17/24 cells tested ($n = 17$, $p < 0.0001$, paired t-test). Sample data records and the time course of the action of NPY are illustrated in Figure 4-1A. In this particular cell, the effect of NPY took about 10 min to develop and recovered over a period of 10 min. However, the time course of action of NPY varied from cell to cell; wash in times ranged from 5 to 10 min and washout times ranged from 10 to 60 min or longer.

The Y1-selective agonist, [F7,P34]NPY (Soll *et al.*, 2001) was tested on evoked EPSCs. [F7,P34]NPY (1 μM) did not affect EPSC amplitude in any of the 6 cells tested ($n = 6$, $p > 0.05$, paired t-test). Figure 4-1B shows the lack of effect of [F7,P34]NPY on the evoked EPSC. By contrast the Y2/Y5 agonist, NPY 13-36 (300 nM), reduced EPSC amplitude by 45.5 ± 5.86% ($n = 8/14$ cells tested, $p < 0.005$). Sample data records are shown in Figure 4-1C. Because NPY 13-36 has been shown to bind to Y5 receptors (Gerald *et al.*, 1996), the selective Y2 agonist [Ahx$^{5-24}$]NPY (Rist *et al.*, 1996; Rist *et al.*, 1997) was also tested on the evoked EPSC. [Ahx$^{5-24}$]NPY (500 nM or 1 μM) reduced the amplitude of the EPSC by 38.6 ± 6.07% ($n = 9/13$ cells tested, $p < 0.005$, paired t-test). An example of this type of experiment is shown in Figure 4-1D.

Figure 4-2 is a summary histogram of the effects of NPY (300 nM-1 μM), NPY-13-36 (300 nM) and [Ahx$^{5-24}$]NPY (1 μM) on the evoked EPSCs.

To characterize further the mechanism by which NPY reduced evoked EPSC amplitude, the effect of NPY on paired-evoked EPSCs was examined. Paired-pulse stimulation can produce either facilitation or depression. Facilitation is thought to reflect an enhancement in neurotransmitter release due to the transient accumulation of Ca^{2+}

close to release sites (Del Castillo & Katz, 1954; Zucker, 1989; Zucker & Regehr, 2002). By contrast, short-term paired-pulse depression is thought to reflect decreased transmitter release from the presynaptic terminal. This may involve depletion of transmitter stores, failure of the action potential to invade the presynaptic terminal, activation of presynaptic autoreceptors or a reduction in activity-dependent I_{Ca} responsible for transmitter release (Zucker & Regehr, 2002). Postsynaptic receptor desensitization (Mennerick & Zorumski, 1996; Trussell *et al.*, 1993) may also be involved. A postsynaptic effect of a neuromodulator is assumed to affect the first and second synaptic responses equally, whereas a presynaptic effect will affect the *ratio* of synaptic current amplitudes. See also Kim & Alger (2001).

In the experiment illustrated in Figure 4-3A, two identical stimuli separated by an inter-stimulus interval (ISI; 50 ms) resulted in a small paired-pulse facilitation of the evoked EPSC. The mean ratio of the amplitude of the paired EPSCs with ISIs of 30-100 ms was 1.62 ± 0.29 ($EPSC_2/EPSC_1$; $n = 7$). In 7 out of 13 cells, superfusion of NPY (300nM) decreased the mean ratio of $EPSC_2/EPSC_1$ to 0.83 ± 0.18 ($n = 7$, $p < 0.05$, paired t-test). These data suggest that NPY may exert its effect via a presynaptic mechanism. In Figure 4-3B, the EPSCs have been normalized to the amplitude of $EPSC_1$ to better illustrate the change in the paired-pulse ratio. Figure 4-3C is a summary histogram demonstrating the effect of NPY (300nM) on the paired-pulse ratio.

Miniature Excitatory Postsynaptic Currents

Because paired-pulse experiments suggested NPY acted presynaptically to reduce transmitter release, the actions of NPY and the Y2 agonist, $[Ahx^{5-24}]$NPY, were examined on TTX-resistant mEPSCs using a CsCl-based internal solution. In the presence of TTX, changes in the frequency of action-potential independent events suggest the effect is via a presynaptic mechanism, whereas a change in event amplitude suggests a postsynaptic action.

The effectiveness of TTX was monitored by its ability to block the evoked EPSC and the effect of NPY (300 nM) on mEPSCs was tested. NPY reduced the mEPSC frequency in five cells tested (n = 5/7 cells tested, Kolmogorov-Smirnov test, $p < 0.05$, Figure 4-4A and B). By contrast, NPY had no effect on mEPSC amplitude in these same neurons (n = 5/7 cells tested, Kolmogorov-Smirnov test, $p > 0.05$, Figure 4-4A and C). This suggests that NPY acted presynaptically to reduce transmitter release.

Similarly, in four cells the Y2 agonist [Ahx$^{5-24}$]NPY (1μM) reduced the frequency tested (n = 4/6 cells tested, Kolmogorov-Smirnov test, $p < 0.05$, Figure 4-5A-C), yet had no effect on mEPSC amplitude in these same cells (n = 4/6 cells tested, Kolmogorov-Smirnov test, $p > 0.05$, Figure 4-5A-C).

NPY and NPY-selective Agonists: Inhibitory Synaptic Transmission

Evoked Inhibitory Synaptic Transmission

IPSCs were evoked in substantia gelatinosa neurons at a holding potential of −70 mV by focal stimulation with a patch electrode unless stated otherwise. Excitatory synaptic responses were blocked by inclusion of the glutamate receptor antagonists, AP5 (50 μM) and CNQX (10 μM) in the aCSF.

Superfusion of NPY (300 nM) reduced IPSC amplitude by an average of 42.7 ± 6.51% in 7/9 cells tested (n = 7, p < 0.0001, paired t-test). Sample data records are illustrated in Figure 4-6A. The Y1-selective agonist, [F7,P34]NPY (Soll *et al.*, 2001) and the Y1/Y5 agonist [Leu31,Pro34]NPY (500 nM) (Gerald *et al.*, 1996) were also tested on the evoked IPSCs. [F7,P34]NPY (1 μM) reduced IPSC amplitude by 52.0 ± 4.67% in 6/12 cells tested (n = 6, p < 0.0002, paired t-test). Similarly, [Leu31,Pro34]NPY reduced IPSC amplitude by 48.1 ± 13.27% in 5/9 cells tested (n = 5, p < 0.01, paired t-test). Figure 4-6B shows the effect of [F7,P34]NPY on the evoked IPSC. By contrast the Y2/Y5 agonist, NPY 13-36 (300 nM) (n = 14) and the selective Y2 agonist [Ahx$^{5\text{-}24}$]NPY (n = 4) did not affect IPSC amplitude. Data records are shown in Figure 4-6C and 4-6D. The effects of [Leu^{31},Pro^{34}]NPY, which were similar to those of [F7, P34]NPY, are not illustrated.

Figure 4-7 is a summary histogram of the effects of NPY (300 nM-1 μM), [Leu^{31},Pro^{34}]NPY (1 μM), [F7,P34]NPY (1 μM), NPY-13-36 (300 nM) and [Ahx$^{5\text{-}24}$]NPY (1 μM) on the evoked IPSCs.

As described in the General Methods, paired-pulse experiments were based on averages of 10-20 sweeps. However, when a series of 10-20 paired-pulse IPSCs was recorded there was considerable inter-trial variability. For example, the first paired stimuli may have exhibited paired-pulse facilitation, whereas the next paired stimuli exhibited paired-pulse depression. When the data of 10-20 sweeps were averaged, the response usually exhibited overall paired-pulse facilitation. In the experiment illustrated in Figure 4-8A, two identical stimuli separated by an inter-stimulus interval (ISI; 100 ms) resulted in paired-pulse facilitation of the evoked IPSC. The mean ratio of the amplitude of the paired IPSCs was 1.22 ± 0.13 ($IPSC_2/IPSC_1$; $n = 5$). In 5 out of 8 cells, superfusion of NPY (300 nM) decreased the mean ratio of $IPSC_2/IPSC_1$ to 0.92 ± 0.13 ($n= 5$, $p < 0.001$, paired t-test). These data suggest that NPY may exert its effect via a presynaptic mechanism. Sample data records are shown in Figure 4-8A. In Figure 4-8B, the IPSCs have been normalized to the amplitude of $IPSC_1$ to better illustrate the change in the paired-pulse ratio. Figure 4-8C is a summary histogram demonstrating the effect of NPY (300 nM) on the paired-pulse ratio.

Miniature Inhibitory Postsynaptic Currents

The effect of NPY (300 nM) on mIPSCs was tested on nine cells and mIPSCs were recorded using a CsCl-based internal solution. In the presence of 50 μM AP5 and 10 μM CNQX, NPY reduced the mIPSC frequency in six cells ($n = 6/9$ cells tested; Kolmogorov-Smirnov test, $p < 0.05$, Figure 4-9A). In of four out of the six cells in which NPY produced changes in mIPSC frequency, NPY had no effect on mIPSC amplitude distributions ($n = 4/9$ cells tested; Kolmogorov-Smirnov test, $p > 0.05$, Figure 4.9B). However, in the remaining two cells NPY reduced mIPSC amplitude and frequency ($n = 2/9$ cells tested, Kolmogorov-Smirnov test, $p < 0.05$; data not shown). These observations suggest, that in some cells, NPY may exert both pre- and postsynaptic actions.

Similarly, the Y1 agonist, [F7, P34]NPY (1 μM), reduced the frequency ($n = 4/7$ cells tested; Kolmogorov-Smirnov test, $p < 0.05$; Figure 4-10A), and reduced the amplitude of the mIPSCs in the cells which responded to [F7, P34]NPY ($n = 4/7$ cells tested; Kolmogorov-Smirnov test, $p < 0.05$; Figure 4-10B). These observations suggest Y1 receptors are located on presynaptic terminals and likely postsynaptically on substantia gelatinosa neurons. These findings are in agreement with recent reports of presynaptic Y1 receptors in dorsal horn (Littlewood *et al.*, 1995; Yamamoto *et al.*, 1994). Whereas, the postsynaptic receptors may correspond to the Y1 receptors localized to the dendrites of local dorsal horn interneurons (Hokfelt *et al.*, 1994; Ji *et al.*, 1994; Xu *et al.*, 1999; Zhang *et al.*, 1994a).

NPY Actions on GABA- and Glycine-Mediated Synaptic Currents

Immunohistochemistry has suggested that NPY-IR is present only in GABAergic interneurons in lamina II (Polgar *et al.*, 1999; Rowan *et al.*, 1993). As well, NPY appeared to increase mIPSC decay times (see Figure 4-9A). Because differences in kinetics of GABA and glycine mediated events have been reported in dorsal horn (Chery & de Koninck, 1999; Grudt & Henderson, 1998; Li *et al.*, 1999), it is possible NPY may have differentially affected GABA- and glycine-mediated events. Therefore, the effect of NPY on pharmacologically-isolated GABAergic IPSCs was compared with its effects on glycinergic IPSCs.

In the presence of strychnine (1μM), AP5 (50 μM) and CNQX (10 μM) focal stimulation elicited bicuculline-sensitive GABAergic IPSCs. In 3/6 cells, superfusion of NPY (300nM) reduced the amplitude of the GABAergic IPSC by 36.5 ± 1.98% ($n = 3$, $p < 0.001$, paired t-test). Raw data traces illustrating the effect of NPY on the GABAergic IPSC are illustrated in Figure 4-11A and a summary histogram of the effect of NPY on the GABAergic IPSC is shown in Figure 4-11B.

In the presence of bicuculline (10μM), AP5 (50 μM) and CNQX (10 μM) focal stimulation elicited strychnine-sensitive glycinergic IPSCs. In 3/6 cells, superfusion of NPY (300nM) reduced the amplitude of the glycinergic IPSC by 38.3 ± 4.24% ($n = 3$, $p < 0.005$, paired t-test). Raw data traces illustrating the effect of NPY on the glycinergic IPSC are illustrated in Figure 4-11C and a summary histogram of the effect of NPY on the glycinergic IPSC is shown in Figure 4-11D. The effect of NPY on GABA- and glycine-mediated IPSCs is consistent with the observation that GABA and glycine are often co-released from interneurons in the spinal cord (Chery & de Koninck, 1999; Jonas *et al.*, 1998; Li *et al.*, 1999).

Actions of Y1 and Y2 Receptor Antagonists on NPY Modulation of Synaptic Currents

If the effect of NPY on IPSCs is mediated via Y1 receptors it should be antagonized by the antagonists BIPB3226 (Rudolf *et al.*, 1994) or BIBO3304 (Wieland *et al.*, 1998). Therefore, we examined the effect of BIBP3226 on NPY-induced suppression of the evoked IPSC. NPY (300nM) suppressed the evoked IPSC by 45.8 ± 14.7% ($n = 2/4$ cells tested). In the presence of the Y1 antagonist, BIBP3226 (1 μM), the effect of NPY on the IPSC was prevented. Sample data are shown in Fig 4-12A. Figure 4-12B shows the time course of the effect of BIBP3226 on NPY-mediated suppression of the evoked IPSC.

If the effect of NPY on the EPSC is mediated via the Y2 receptor, it should be antagonized by a Y2 receptor antagonist. Recently, a selective non-peptide Y2 receptor antagonist, (S)-N^2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-yl]-1-

piperazinyl]-2-oxoethyl] cylopentyl] acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2, 4-triazol-4-yl]ethyl]-argininamid (BIIE0246), was synthesized (Doods *et al.*, 1999) and it has high affinity for the Y2 receptor in the CNS and PNS (Dumont *et al.*, 2000). BIIE0246 blocks the effects of NPY at sympathetic nerve terminals (Smith-White *et al.*, 2001) and in the hippocampus acts at presynaptic Y2 receptors to prevent the inhibitory actions of NPY (El Bahh *et al.*, 2002; Weiser *et al.*, 2000). Therefore, we studied the effect of BIIE0246 against the action of NPY on the evoked EPSC in substantia gelatinosa neurons. NPY (300nM) suppressed the evoked EPSC by 42.9 ± 6.52% in all cells tested (n = 4). In the presence of the Y2 antagonist BIIE0246 (100 nM), the effect of NPY on the EPSC was prevented. Sample data are shown in Fig 4-13A. Figure 4-13B shows the time course of the effect of BIIE0246 on NPY-mediated suppression of the evoked IPSC. In addition, in two cells BIIE0246 affected the kinetics of the evoked EPSC. Because BIIE0246 has been shown to be highly lipophilic, it may interact with ion channels or receptors in the presynaptic membrane and may explain some of the kinetic changes in the EPSC. Alternatively, it may reflect a tonic action of NPY in the spinal cord dorsal horn.

Comparison of the Actions of NPY with the μ-opioid Agonist, DAMGO

Because we were interested in the potential analgesic actions of NPY (Taiwo & Taylor, 2002),(Naveilhan *et al.*, 2001) its actions were compared with the μ-opioid agonist, DAMGO.

The effects of NPY and DAMGO on the evoked EPSC were similar. NPY reduced the amplitude of the evoked EPSC by 45.5 ± 4.64% ($n = 17/24$; $p < 0.0001$, paired t-test). Sample data records and the time course of the actions of NPY are illustrated in Figure 4-14A and C. Similarly, DAMGO reduced the evoked EPSC by 59.4 ± 7.35% ($n = 9/9$, $p < 0.0005$, paired t-test). Sample data records and the time course of the actions of DAMGO are illustrated in Figure 4-14B and C. Note the data in Figure 4-14A-C were obtained from the same cell. This suggests NPY and μ-opioid receptors are likely both present on the same primary afferent terminals. Figure 4-14D is a summary histogram illustrating the similarity in EPSC suppression by NPY and DAMGO.

For cells tested with both NPY and DAMGO, DAMGO reduced EPSC amplitude in *every* cell tested ($n = 9/9$), whereas NPY reduced EPSC amplitude in *almost* all of the cells tested ($n = 7/9$). Figure 4-14E shows the total number of cells that responded to NPY, DAMGO or DAMGO and NPY.

Postsynaptic Actions of NPY

NPY activates an inwardly-rectifying K^+ conductance in amphibian sympathetic neurons (Zidichouski *et al.*, 1990) and in mammalian hypothalamic (Sun & Miller, 1999) and thalamic neurons (Sun *et al.*, 2001). The postsynaptic actions of NPY were therefore examined. NPY (300 nM-1 μM; $n = 14/53$ cells) activated an inwardly-rectifying conductance which was reflected by a 49.0 ± 5.98 pA increase in current at −140 mV (Figure 4-14 A and C). This was very similar to the actions of NPY on hypothalamic (Sun & Miller, 1999), thalamic (Sun *et al.*, 2001) and amphibian sympathetic neurons (Zidichouski *et al.*, 1990). Similarly, DAMGO (1 μM) activated an inwardly-rectifying K^+ current of 60.5 pA ± 10.0 pA ($n = 8/32$ cells tested) at −140 mV (Figure 4-15 B and C) which confirms previous reports (Grudt & Williams, 1994; Schneider *et al.*, 1998).

The magnitude of the current activated by NPY or by DAMGO was not statistically different (Figure 4-15C) and the number of cells that responded to either ligand was similar (Figure 4-15D). Interestingly, only 2/27 cells responded to both NPY and DAMGO, suggesting that there may be differential expression of NPY receptors and μ-opioid receptors on dorsal horn neurons.

Discussion

The main findings of this study are that NPY acts at a presynaptic Y2 receptor to attenuate excitatory synaptic transmission and at a presynaptic Y1 receptor to attenuate inhibitory synaptic transmission in rat substantia gelatinosa. Postsynaptic Y1 receptors may also be involved in this effect on inhibitory transmission, but no evidence was found for involvement of a postsynaptic Y2 receptor in attenuation of excitatory transmission. In addition, NPY suppressed GABAergic and glycinergic inhibitory transmission equally. The presynaptic effect of NPY on excitatory transmission was similar to the μ-opioid agonist, DAMGO, both in terms of efficacy and site of action; NPY and μ-opioid receptors were frequently co-localized on the same presynaptic terminals. NPY also acted on postsynaptic receptors and activated an inwardly-rectifying conductance. The magnitude of the NPY response was similar to DAMGO. However, NPY and μ-opioid receptors were infrequently co-localized on the postsynaptic membrane.

The finding that presynaptic Y2-receptor activation suppressed EPSCs is supported by the paired-pulse data with NPY and by the effect of NPY and the Y2-selective agonist, [Ahx$^{5-24}$]NPY, on mEPSC frequency but not amplitude distribution. This is consistent with binding and *in situ* hybridization studies that show Y2 receptor expression on primary afferent terminals innervating lamina II (Mantyh *et al.*, 1994; Zhang *et al.*, 1995). Our observation is also consistent with Y2 suppression of glutamate release from spinal cord synaptosomes (Martire *et al.*, 2000) and inhibition of EPSPs by NPY and the related peptide, PYY, in lamprey spinal cord (Parker *et al.*, 1998; Parker, 2000; Ullström *et al.*, 1999).

The change in paired-pulse ratios for our evoked EPSC experiments provided evidence that NPY acted presynaptically. A postsynaptic effect is assumed to affect the first and second synaptic responses equally, whereas a presynaptic effect will affect the *ratio* of synaptic current amplitudes (Del Castillo & Katz, 1954; Zucker & Regehr, 2002). Therefore, these actions of NPY on transmitter release may explain its ability to reduce Substance P release in the substantia gelatinosa (Duggan *et al.*, 1991).

Although the mechanism of this presynaptic Y2 effect remains to be determined, it is noteworthy that Y2 receptors are found on the cell bodies of small DRG neurons and these receptors are negatively coupled, via G-proteins, to N-type calcium channels (Abdulla & Smith, 1999; Bleakman *et al.*, 1991; Walker *et al.*, 1988). The present experiments suggest that NPY may be modulating other processes, such as transmitter mechanisms, at the primary afferent terminal. This differs from sympathetic neuron terminals where NPY has been shown to decrease Ca^{2+} influx through N-type calcium channels and presumably decreases transmitter release (Toth *et al.*, 1993).

The effect of NPY and the Y2-agonist, [Ahx^{5-24}]NPY, on mEPSC frequency, but not amplitude distributions, further supports that NPY acts at a presynaptic locus. Because these experiments were done in the presence of TTX, it is unlikely that the decrease in frequency was due to inhibition of presynaptic Ca^{2+} channels by NPY. This suggests that NPY may affect other mechanisms at the terminal. One possibility is that NPY may modulate the vesicle release machinery, as occurs in the arcuate nucleus of the hypothalamus (Rhim *et al.*, 1997). It should also be mentioned that the mEPSCs may have originated from primary afferent terminals, as well as from local interneurons or descending inputs (Bongianni *et al.*, 1990; Doyle & Maxwell, 1993; Holets *et al.*, 1988). Thus, extrapolation of the effects of NPY on Ca^{2+} channels in DRG cell bodies may not be entirely relevant to understanding the presynaptic actions of NPY on mEPSCs in substantia gelatinosa neurons

The finding that presynaptic Y1 receptors suppressed inhibitory synaptic transmission is supported by the paired-pulse experiments with NPY and the effect of the Y1-selective agonist, [F7, P34]NPY, on mIPSCs. The mIPSC frequency decrease caused by [F7, P34]NPY is consistent with recent reports of presynaptic Y1 receptors in the dorsal horn (Bao *et al.*, 2002; Brumovsky *et al.*, 2002). In some cells, NPY and [F7, P34]NPY decreased mIPSC amplitude distributions, suggesting an additional postsynaptic action. This is consistent with reports that the Y1 receptor is localized to the dendrites of somatostatin (SST)-positive dorsal horn neurons (Zhang *et al.*, 1994a; Zhang *et al.*, 1999). SST-containing neurons in the dorsal horn represent a morphologically heterogeneous population that is comprised of both stalked and islet cells (Alvarez & Priestley, 1990; Ribeiro-da-Silva & Cuello, 1990; Todd & Spike, 1993), some of which are thought to be excitatory interneurons (Spike & Todd, 1992; Todd & McKenzie, 1989). Although the mechanism by which NPY or [F7, P34]NPY affected mIPSC amplitude is unclear, it may have activated an inwardly-rectifying K^+ conductance in the dendrites of these substantia gelatinosa neurons. This hypothesis is consistent with studies showing the presence of GIRK channels in the distal dendrites of hippocampal (Kemp *et al.*, 1996; Pasternak, 1993) and cortical neurons (Gobel, 1978), where they may modulate dendritic excitability. Even though a CsCl-based internal solution was used for recording mIPSCs, it may not have fully dialysed into the dendrites thus incompletely blocking the GIRK channels. Another possibility may involve cross-talk between the Y1 receptor and postsynaptic $GABA_A$ and/or glycine receptors, which has been described for dopamine and melatonin GPCRs and $GABA_A$ (Liu *et al.*, 2000; Wan *et al.*, 1999).

NPY suppressed GABAergic and glycinergic inhibitory transmission similarly in lamina II neurons. The receptor subtype mediating this effect was not determined, but was likely acting via the Y1 receptor, as described above. NPY is restricted to GABAergic neurons in lamina II (Polgar *et al.*, 1999; Rowan *et al.*, 1993). It is unknown if NPY receptors are differentially expressed on GABAergic or glycinergic neurons, but the ability of NPY to suppress both GABA- and glycine-mediated currents is consistent with studies that show these neurotransmitters are often co-localized and co-released from synapses in the superficial dorsal horn (Chery & de Koninck, 1999; Li *et al.*, 1999; Todd *et al.*, 1996).

The actions of NPY and Y2 agonists on excitatory synaptic transmission were quantitatively very similar to those of the μ-opioid, DAMGO. The effect of DAMGO on evoked EPSCs is in agreement with previous findings (Glaum *et al.*, 1994; Hori *et al.*, 1992; Knoflach *et al.*, 1996; Kohno *et al.*, 1999). Interestingly, most primary afferent terminals innervating lamina II co-expressed NPY and the Y2 and μ-opioid receptor. No systematic studies have been performed which show co-expression of NPY and μ-opioid receptors in dorsal root ganglia or on primary afferent terminals. However, several lines of evidence support the hypothesis that these receptors are indeed co-localized. First, NPY and opioid receptor binding sites in the superficial dorsal horn are reduced after dorsal rhizotomy or by capsaicin treatment (Gouarderes *et al.*, 1985; Hohmann *et al.*, 1999; Hohmann & Herkenham, 1998; Kar & Quirion, 1992; Morris & Herz, 1987). Second, NPY and morphine inhibit the release of Substance P from nociceptive primary afferents (Duggan *et al.*, 1991; Jessell & Iversen, 1977; Yaksh *et al.*, 1980) but also see (Trafton *et al.*, 1999). Third, NPY and opioid agonists both produce analgesia when applied intrathecally or intracerebroventricularly (Broqua *et al.*, 1996; Hua *et al.*, 1991; Kerr *et al.*, 1998; Kruger, 1992; Seltzer *et al.*, 1991; Xu *et al.*, 1994). Finally, local interneurons expressing GABA and NPY synapse onto neurons expressing the NK1 receptor, providing an anatomical basis for the analgesic effect of NPY (Polgar *et al.*, 1999).

The Y2-mediated suppression of excitatory transmission is similar to DAMGO and provides a cellular mechanism for the analgesic effect of NPY. However, Y1 receptor agonists suppress inhibitory transmission but still produce analgesia, which is difficult to reconcile. Moreover, DAMGO also appears to have similar effects on IPSCs in dorsal horn (Grudt & Henderson, 1998; Marinelli *et al.*, 2002; Moran & Smith, 2002). Thus, the effects of NPY and Y1 agonists on inhibitory transmission are consistent with the role of Y1 receptor activation in antinociception. In intact and axotomized rats, intrathecally administered Y1 agonists inhibit the flexor reflex (Xu *et al.*, 1999) and i.c.v. application of Y1 agonists have an anti-nociceptive action in the mouse writhing test (Broqua *et al.*, 1996). In addition, Y1 receptor activation has an antihyperalgesic action in an inflammatory pain model (Taiwo & Taylor, 2002). Finally, mice lacking the Y1 receptor have reduced nociceptive thresholds for a variety of pain modalities, including thermal, visceral and chemical pain, and have increased neuropathic pain. As a final point, NPY or Y1 agonists do not produce analgesia in these mice (Naveilhan *et al.*, 2001).

The non-peptide Y1 antagonist, BIBP3226, blocked the actions of NPY on the evoked IPSC (1 μM). This observation and the effects of the Y1 agonist, [F7, P34]NPY, and Y1/Y5 agonist, [Leu^{31},Pro^{34}]NPY, provide strong evidence for a Y1 receptor-mediated suppression of inhibitory synaptic transmission in lamina II. The ability of BIBP3226 to antagonize the effect of NPY on the IPSC is consistent with its actions in the paraventricular nucleus of the hypothalamus (PVN) (Pronchuk *et al.*, 2002). The potent and highly selective, non-peptide Y2 receptor antagonist, BIIE0246 (Doods *et al.*, 1999), was tested on the evoked EPSC. BIIE0246 (100 nM) antagonized the effect of NPY on the evoked EPSC. However, in two of the cells tested BIIE0246 affected the kinetics of the EPSC. The mechanism of this effect is unknown, but may be related to the highly lipophilic nature of BIIE0246 (El Bahh *et al.*, 2002). Clearly the actions of BIIE0246 on the EPSC need to be examined further to determine if this effect is reproducible or only an artifact.

Both NPY and DAMGO activated an inwardly-rectifying K$^+$ conductance (GIRK) in substantia gelatinosa neurons. The effects of NPY were similar to those described in thalamus (Sun *et al.*, 2001), hypothalamus (Sun & Miller, 1999) and amphibian sympathetic ganglia (Zidichouski *et al.*, 1990). Likewise, the effects of DAMGO on

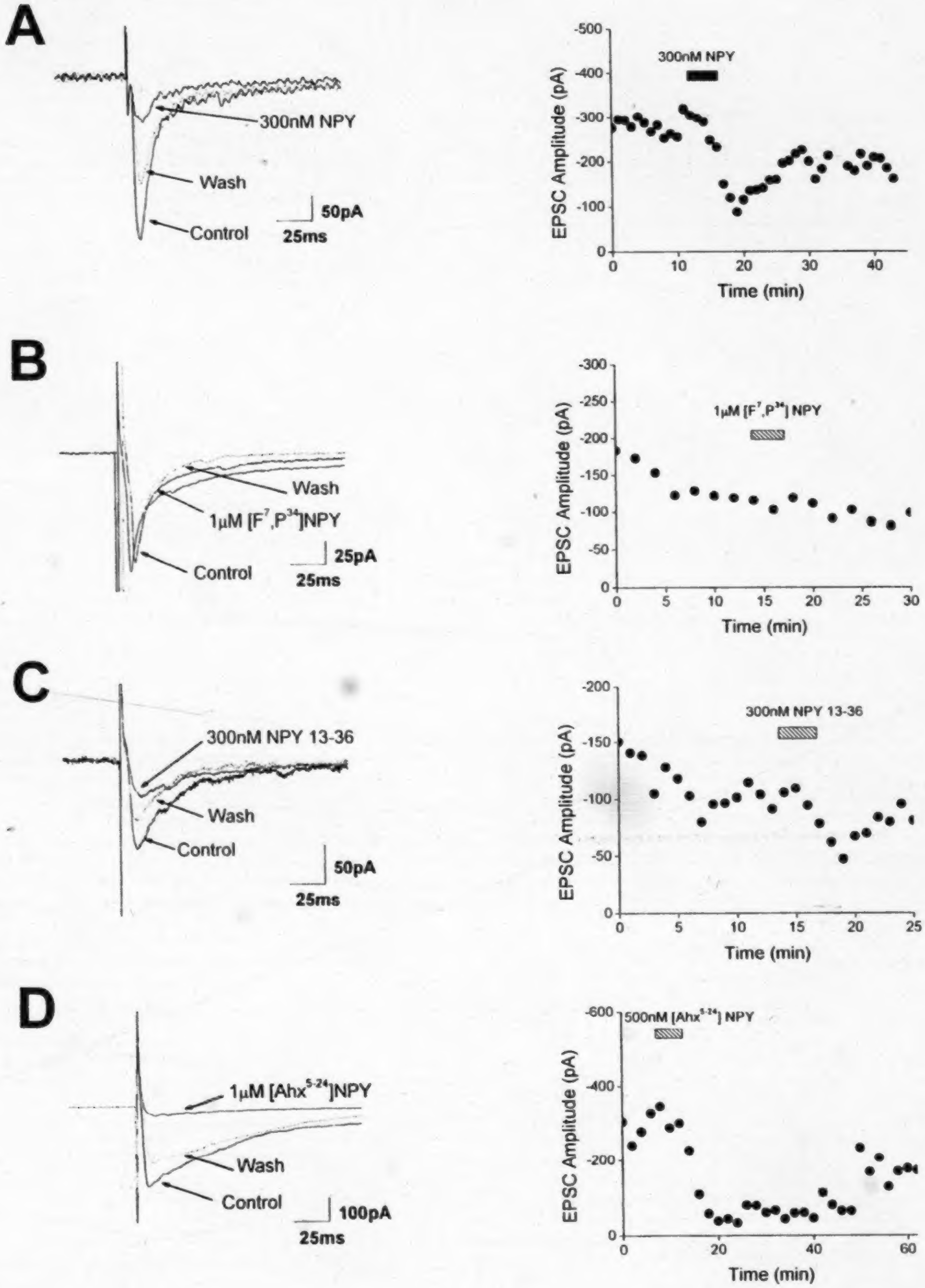
GIRK were in agreement with previous findings of its actions in the dorsal horn (Grudt & Williams, 1994; Schneider *et al.*, 1998). Furthermore, each agonist activated a current of similar magnitude in approximately 25% of the cells tested. However, unlike the effects on synaptic transmission, cells that expressed postsynaptic NPY receptors did not respond to DAMGO and vice versa, suggesting that NPY and μ-opioid receptors are not co-localized on the postsynaptic membrane. Although no studies have systematically determined if NPY and μ-opioid receptors are co-expressed in lamina II neurons, some evidence suggests that they are unlikely to be co-expressed. First, μ-receptors are only found on neurons in lamina II which do not contain GABA or glycine (Kemp *et al.*, 1996), while NPY appears to be restricted to primarily GABAergic neurons (Rowan *et al.*, 1993).

The effect of NPY on synaptic transmission in the dorsal horn, and its similarity to the cellular actions of opioids, supports its role as an analgesic. However, the exact role NPY plays in neuropathic pain is unclear (Hirakawa *et al.*, 2000). NPY has pro- and anti-nociceptive actions (Broqua *et al.*, 1996; Christie *et al.*, 2000; Dickenson & Le Bars, 1983; Hua *et al.*, 1991; Mitchell *et al.*, 1998; Taiwo & Taylor, 2002; Xu *et al.*, 1994). It also has a biphasic dose-effect curve with pro-nociceptive actions at low doses and anti-nociceptive actions at high doses (Xu *et al.*, 1999; Xu *et al.*, 1994). However, the analgesic effect of NPY has been proposed to be mediated via Y1 receptors in intact rats, while Y1 and Y2 mediate its anti-nociceptive actions after axotomy (Xu *et al.*, 1999). Further support for Y1-mediated anti-nociception is provided by Y1 receptor knockout mice which have increased pain responses to a variety of modalities before and after nerve injury (Naveilhan *et al.*, 2001).

In summary, NPY and receptor-specific agonists potently modulate synaptic transmission in the substantia gelatinosa. Y1 receptors selectively suppress inhibitory transmission by pre- and postsynaptic mechanisms, while Y2 receptors suppress excitatory transmission by a presynaptic mechanism. NPY also activates an inwardly-rectifying conductance in a population of substantia gelatinosa neurons. These effects of NPY are comparable to the actions of μ-opioids and suggest NPY has promise as an analgesic for the treatment of neuropathic pain.

Figure 4-1. Neuropeptide Y and Y2 agonists, but not Y1 agonists suppress evoked EPSCs in substantia gelatinosa neurons. EPSCs were evoked by stimulating the dorsal root or dorsal root entry zone in the presence of 10 μM bicuculline and 1 μM strychnine. Left-hand panels illustrate averaged current traces and right-hand panels are graphs to illustrate time course of effect and washout of agonists. A, averaged traces (n = 3) of evoked EPSCs before, during and after application of NPY (300 nM). B, averaged traces (n = 6) of evoked EPSCs before, during and after application of the Y1-selective agonist [F7, P34]NPY (1 μM). Note that [F7, P34]NPY does not affect EPSC amplitude. C, averaged traces (n = 6) of evoked EPSCs before, during and after application of the Y2/Y5 agonist, NPY 13-36 (300 nM). D, averaged traces (n = 6) before, during and after application of the Y2-selective agonist, [Ahx$^{5-24}$]NPY (1 μM). Note that [Ahx$^{5-24}$]NPY strongly suppresses the evoked EPSC. Neurons were voltage clamped at a holding potential of –70 mV, with a CsCl-based internal solution in the recording electrode. Cells shown in C and D suggest that the reduction in EPSC amplitude produced by NPY is mediated via the Y2 receptor.

Figure 4-1



A
300nM NPY
Wash
Control
50pA
25ms
300nM NPY
EPSC Amplitude (pA)
-500
-400
-300
-200
-100
0
0
10
20
30
40
Time (min)
B
Wash
1μM [F7,P34]NPY
Control
25pA
25ms
1μM [F7,P34] NPY
EPSC Amplitude (pA)
-300
-250
-200
-150
-100
-50
0
0
5
10
15
20
25
30
Time (min)
C
300nM NPY 13-36
Wash
Control
50pA
25ms
300nM NPY 13-36
EPSC Amplitude (pA)
-200
-150
-100
-50
0
0
5
10
15
20
25
Time (min)
D
1μM [Ahx5-24]NPY
Wash
Control
100pA
25ms
500nM [Ahx5-24] NPY
EPSC Amplitude (pA)
-600
-400
-200
0
0
20
40
60
Time (min)

Figure 4-2. Summary histogram of the change in evoked EPSC amplitude in response to NPY, Y1 and Y2 agonists. Note that NPY (300 nM and 1 μM), the Y1/Y5 agonist, NPY 13-36 (300 nM), and the Y2-selective agonist, [Ahx$^{5-24}$]NPY (1 μM) reduce evoked EPSC amplitude by approximately 50% (* = $p < 0.0001$; ** = $p < 0.005$). The Y1 agonist, [F7, P34]NPY, was ineffective.

Figure 4-2

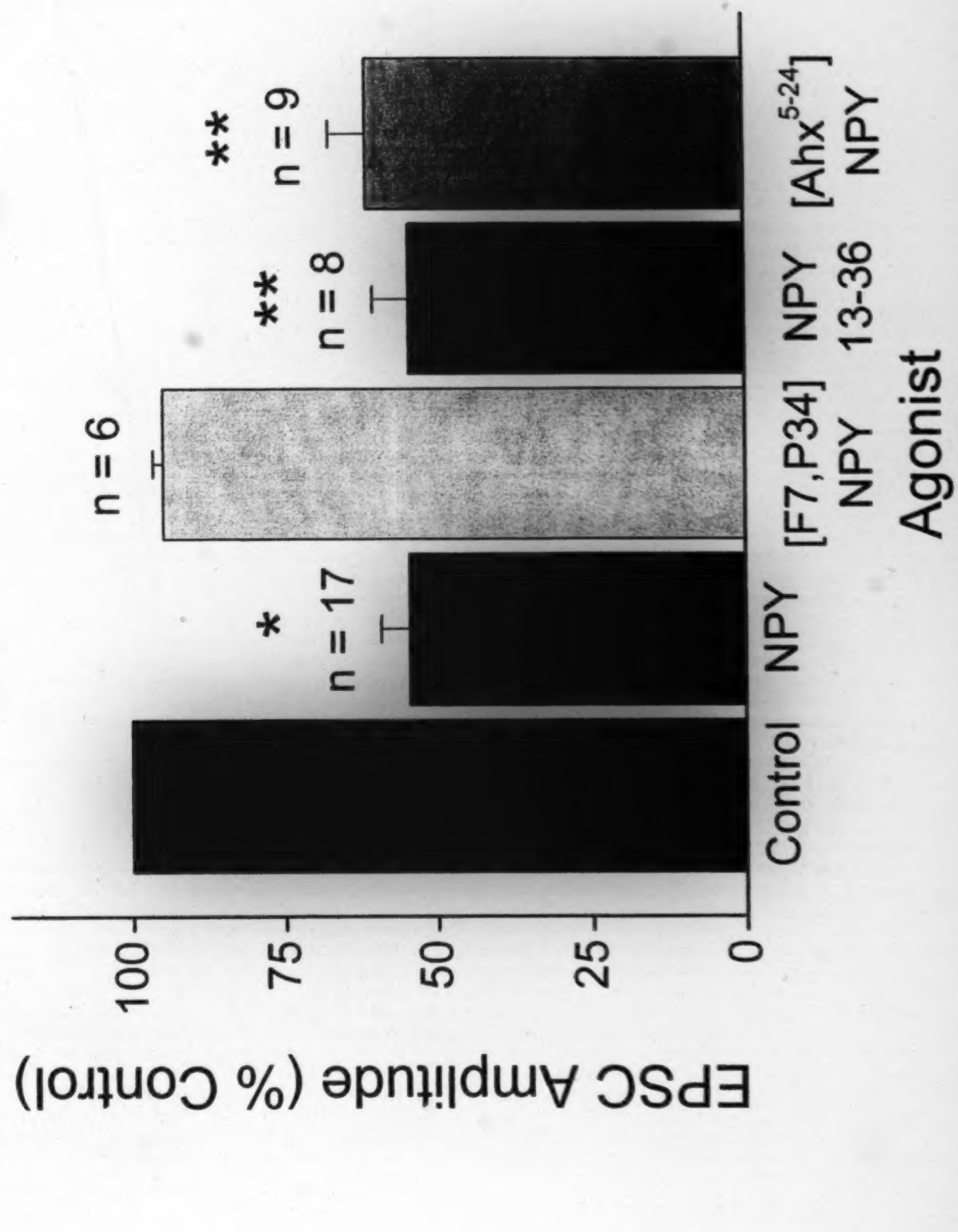

EPSC Amplitude (% Control)
100
75
50
25
0
Control
NPY
[F7,P34] NPY
NPY 13-36
[Ahx$^{5-24}$] NPY
Agonist
*
n = 17
n = 6
**
n = 8
**
n = 9

Figure 4-3. Suppression of the EPSC by NPY is accompanied by a change in the paired-pulse ratio. EPSCs were evoked at a holding potential of –70 mV, with a CsCl-based internal solution in the recording electrode, by dorsal root or dorsal root entry zone stimulation in the presence of 10 μM bicuculline and 1 μM strychnine. A, averaged traces (left panel; n = 10) of pairs of evoked EPSCs (50 ms inter-stimulus interval). NPY (300 nM) (right panel) reducea the amplitude of the evoked EPSCs and produces a marked change in the paired-pulse ratio. B, the change in the paired-pulse ratio caused by NPY is better observed after normalizing the data traces in A to the amplitude of the first EPSC. C, summary histogram of the change in paired-pulse ratio ($EPSC_2/EPSC_1$) induced by NPY (n = 7).

Figure 4-3

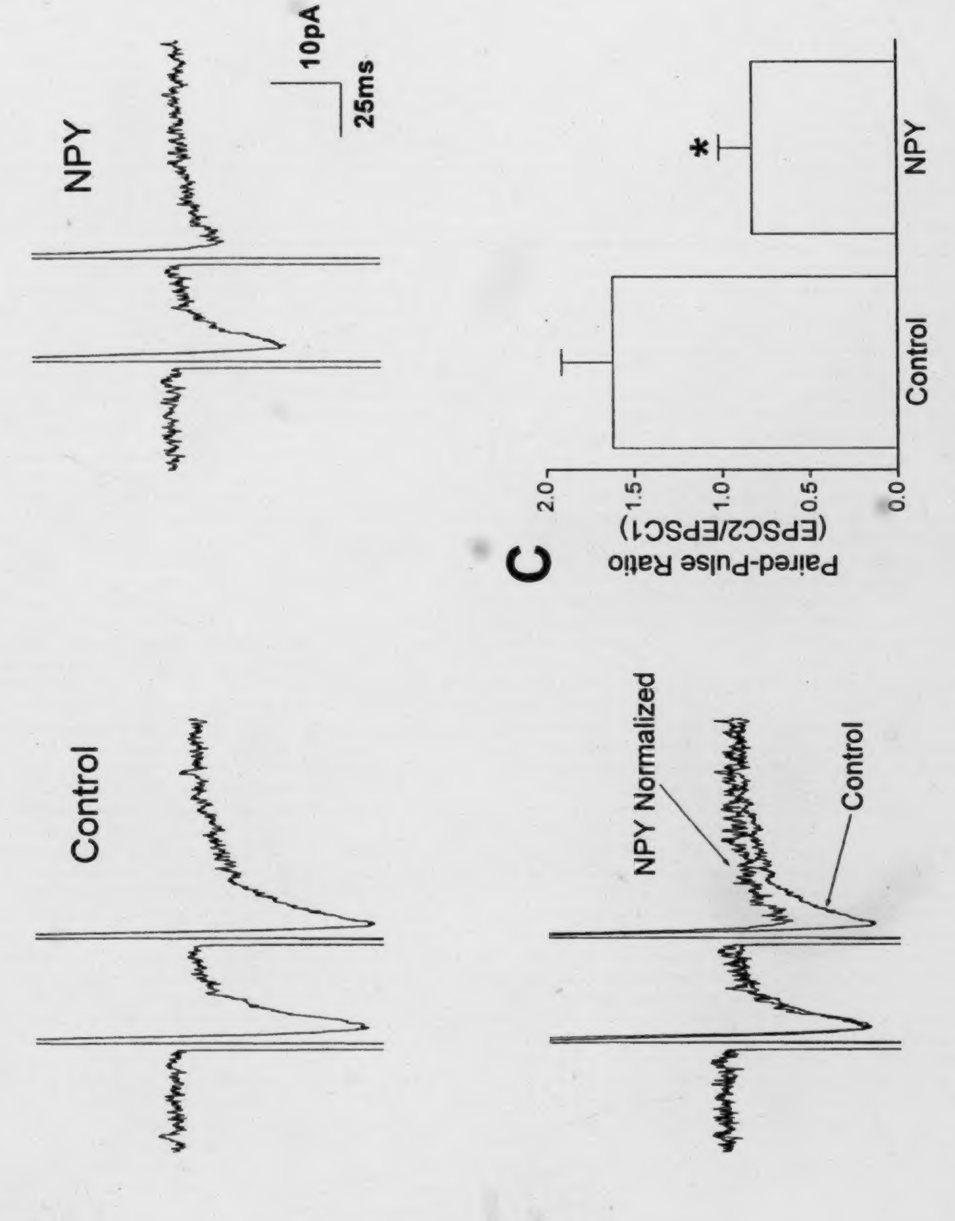

A
Control
NPY
10pA
25ms
B
NPY Normalized
Control
C
Paired-Pulse Ratio
(EPSC2/EPSC1)
2.0
1.5
1.0
0.5
0.0
Control
NPY
*

Figure 4-4. A, mEPSC traces before and during the application of NPY (300 nM). B and C, cumulative fraction plots of the mEPSC interevent interval and amplitude distribution. NPY significantly increased the interevent interval without affecting the amplitude distribution. The neuron was voltage clamped at a holding potential of –70 mV with a CsCl-based internal solution in the recording electrode.

Figure 4-4

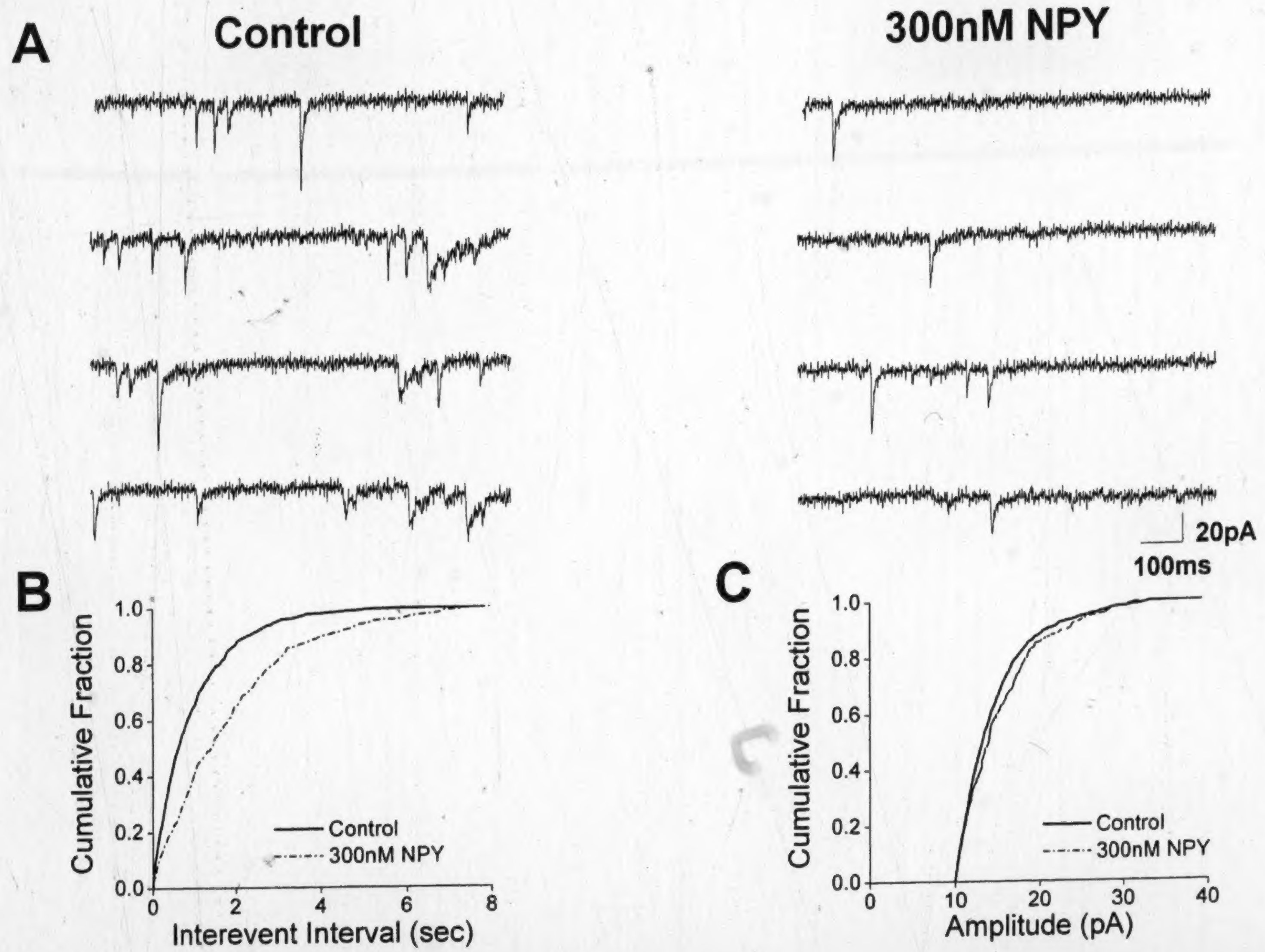

A
Control
300nM NPY
20pA
100ms
B
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Interevent Interval (sec)
Control
300nM NPY
C
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Amplitude (pA)
Control
300nM NPY

Figure 4-5. A, mEPSC traces before and during the application of the Y2-selective agonist, [Ahx$^{5\text{-}24}$]NPY (1 μM). B and C, cumulative fraction plots of the mEPSC interevent interval and amplitude distribution. [Ahx$^{5\text{-}24}$]NPY significantly increased the interevent interval without affecting the amplitude distribution. The neuron was voltage clamped at a holding potential of –70 mV, with a CsCl-based internal solution in the recording electrode.

Figure 4-5

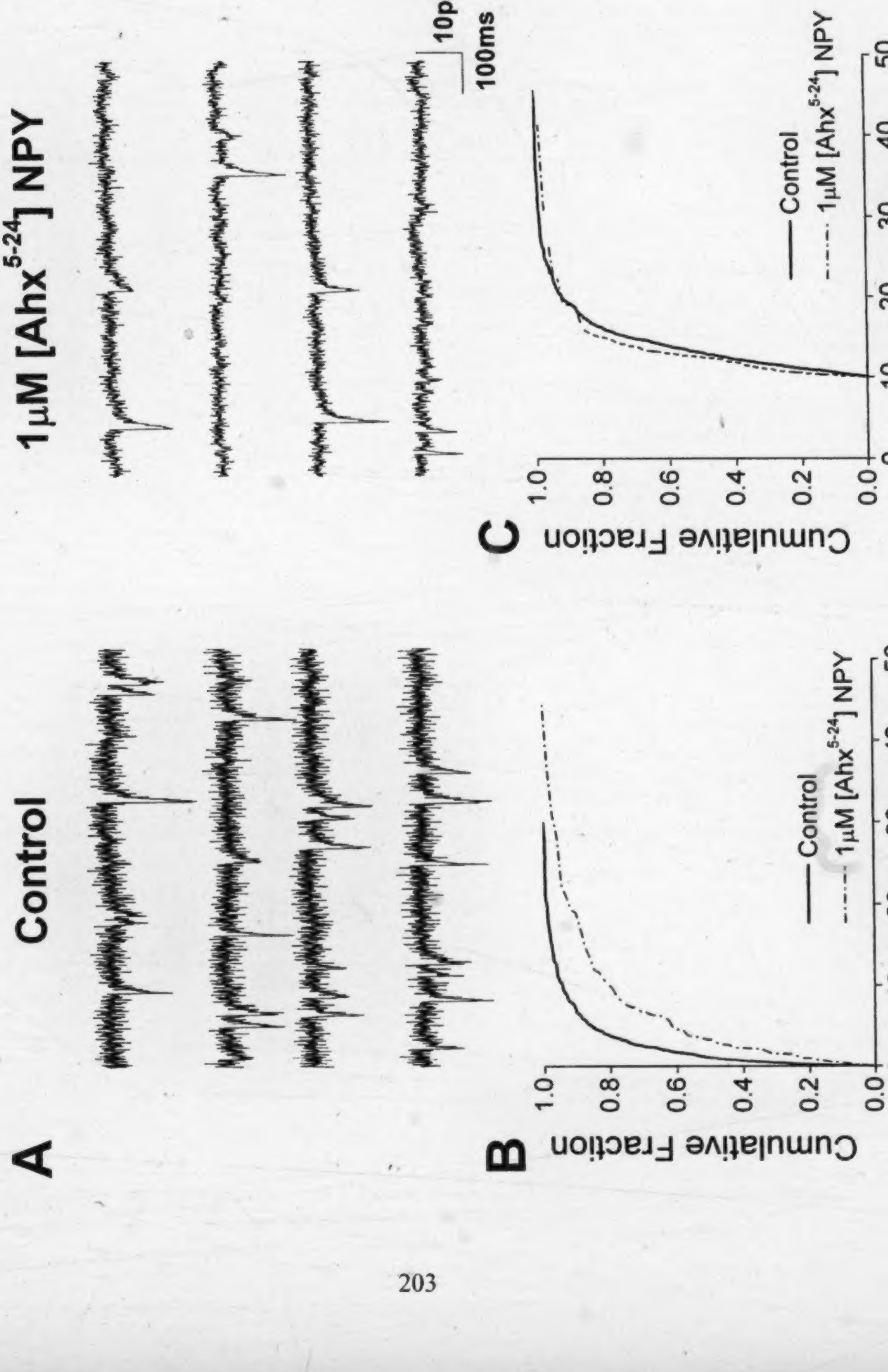

A
Control
1μM [Ahx5-24] NPY
10pA
100ms
B
Cumulative Fraction
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
Interevent Interval (sec)
Control
1μM [Ahx5-24] NPY
C
Cumulative Fraction
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
Amplitude (pA)
Control
1μM [Ahx5-24] NPY

Figure 4-6. Neuropeptide Y and Y1 agonists, but not Y2 agonists suppress evoked IPSCs in substantia gelatinosa neurons. IPSCs were evoked by focal stimulation with a patch electrode in the presence of 50 μM AP5 and 10 μM CNQX. Left-hand panels illustrate averaged current traces and right-hand panels are graphs to illustrate time course of effect and washout of agonists. A, averaged traces (n = 6) of evoked IPSCs before, during and after application of NPY (300nM). B, averaged traces (n = 6) of evoked EPSCs before, during and after application of the Y1-selective agonist [F7, P34]NPY (1 μM). Note that [F7, P34]NPY strongly suppresses the evoked IPSC. C, averaged traces (n =6) of evoked IPSCs before, during and after application of the Y2/Y5 agonist, NPY 13-36 (300 nM). Note that NPY 13-36 does not affect IPSC amplitude. D, averaged traces (n = 6) before during and after application of the Y2-selective agonist, [Ahx$^{5-24}$]NPY (1 μM). Note that [Ahx$^{5-24}$]NPY does not affect IPSC amplitude. Neurons were voltage clamped at a holding potential of –70 mV, with a CsCl-based internal solution in the recording electrode. The cell shown in B suggests that the reduction in IPSC amplitude produced by NPY is mediated via the Y1 receptor.

Figure 4-6

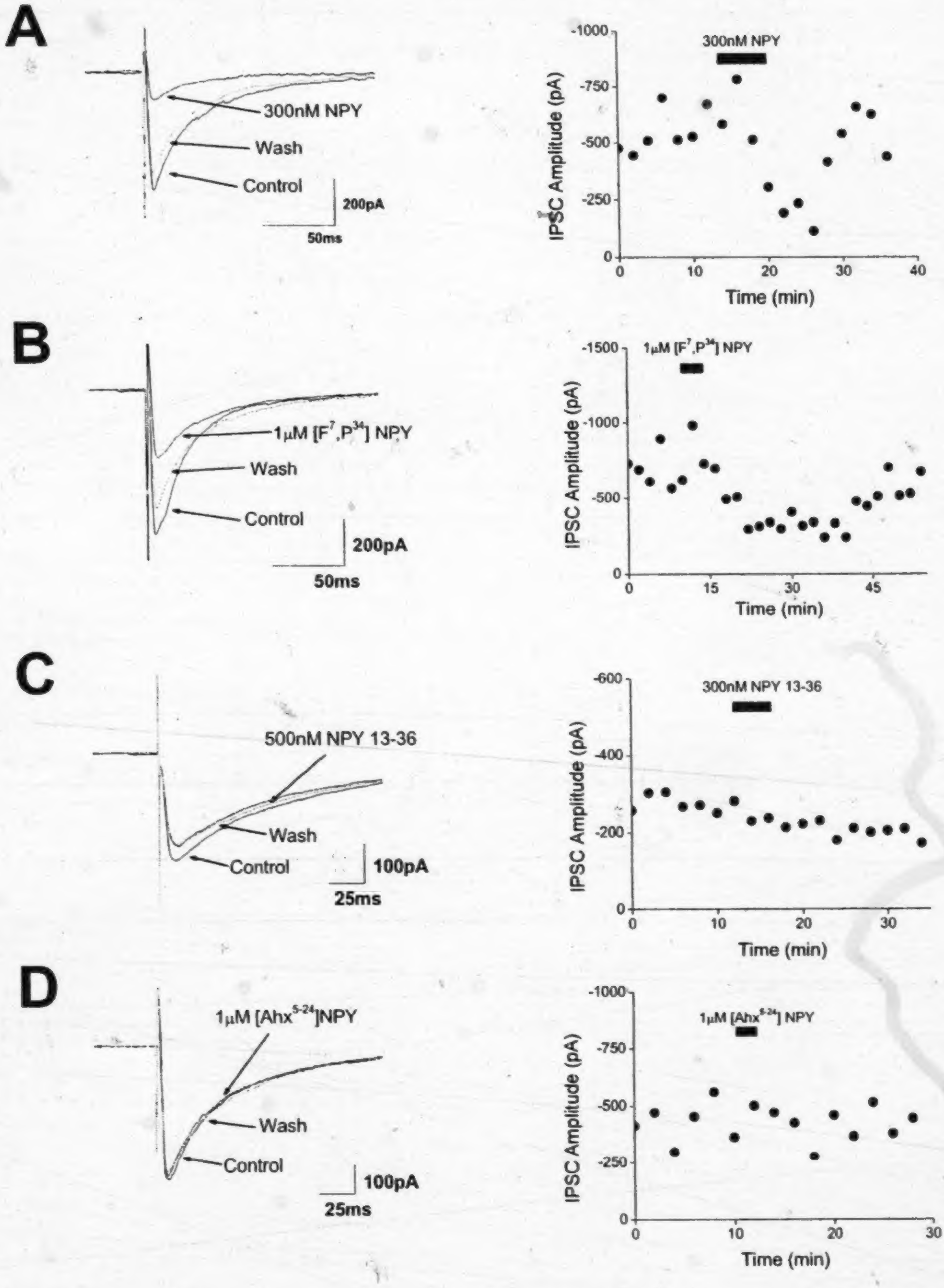

A
300nM NPY
Wash
Control
200pA
50ms
-1000
-750
-500
-250
0
IPSC Amplitude (pA)
0
10
20
30
40
Time (min)
300nM NPY
B
1μM [F^7,P^{34}] NPY
Wash
Control
200pA
50ms
-1500
-1000
-500
0
IPSC Amplitude (pA)
0
15
30
45
Time (min)
1μM [F^7,P^{34}] NPY
C
500nM NPY 13-36
Wash
Control
100pA
25ms
-600
-400
-200
0
IPSC Amplitude (pA)
0
10
20
30
Time (min)
300nM NPY 13-36
D
1μM [Ahx$^{5-24}$]NPY
Wash
Control
100pA
25ms
-1000
-750
-500
-250
0
IPSC Amplitude (pA)
0
10
20
30
Time (min)
1μM [Ahx$^{5-24}$] NPY

Figure 4-7. Summary histogram of the change in evoked IPSC amplitude in response to NPY, Y1 and Y2 agonists. Note that NPY (300nM) and the Y1 agonists, [F7,P34]NPY (1 μM) and [Leu^{31},Pro^{34}]NPY (1 μM) reduced evoked IPSC amplitude by approximately 50% (* = $p < 0.0001$; ** = $p < 0.0002$; *** = $p < 0.01$). The Y2/Y5 agonist, NPY 13-36 (300 nM), and the Y2 agonist, [Ahx$^{5-24}$]NPY (1 μM), were ineffective.

Figure 4-7

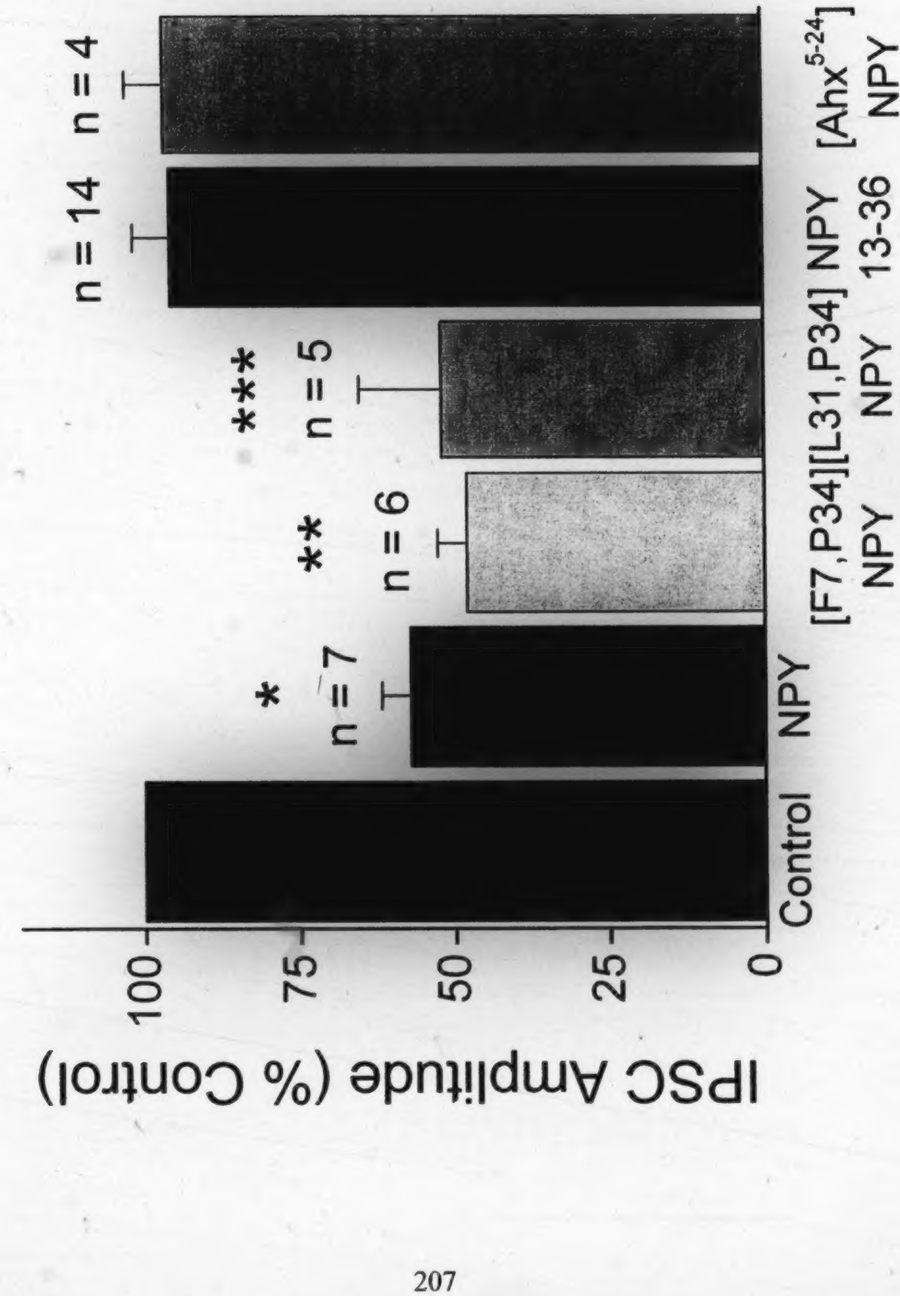

IPSC Amplitude (% Control)
100
75
50
25
0
Control
NPY
[F7,P34] NPY
[L31,P34] NPY
NPY 13-36
[Ahx5-24] NPY
Agonist
*
n = 7
**
n = 6
***
n = 5
n = 14
n = 4

Figure 4-8. Suppression of the IPSC by NPY is accompanied by a change in the paired-pulse ratio. IPSCs were evoked at a holding potential of 0 mV (K^+-gluconate-based internal solution in the patch pipette) by focal stimulation with a patch electrode in the presence of 50 μM AP5 and 10 μM CNQX. A, averaged traces (left panel; n = 12) of pairs of evoked IPSCs (50 ms inter-stimulus interval). NPY (300 nM) (right panel) reduces the amplitude of the evoked IPSCs and changes the paired-pulse ratio. B, the change in paired-pulse ratio is better observed after normalizing the data traces in A to the amplitude of the first IPSC. C, summary histogram of the change in paired-pulse ratio ($IPSC_2/IPSC_1$) induced by NPY (n = 5, * $p < 0.001$).

Figure 4-8

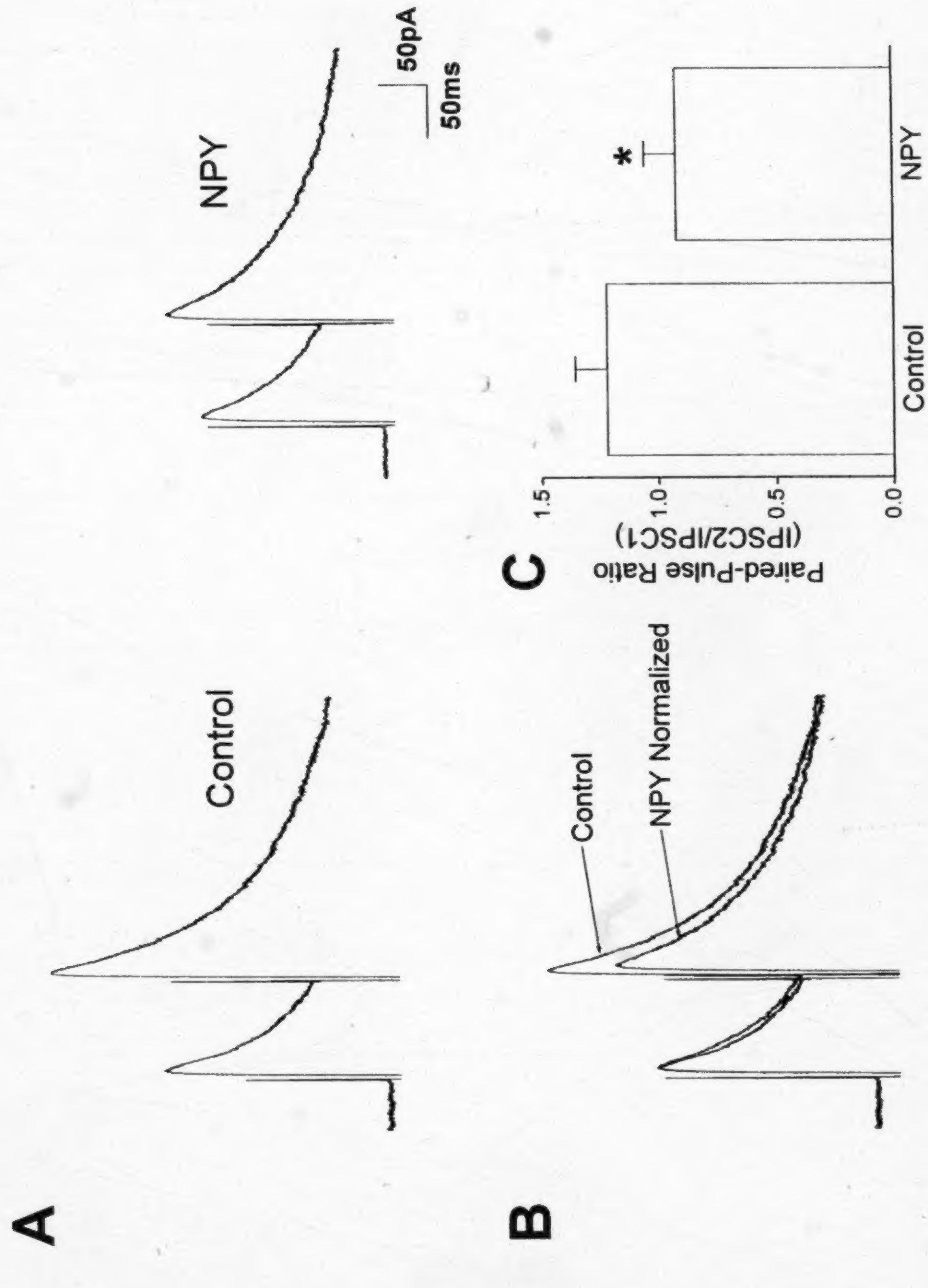

A
Control
NPY
50pA
50ms
B
Control
NPY Normalized
C
Paired-Pulse Ratio
(IPSC2/IPSC1)
1.5
1.0
0.5
0.0
*
Control
NPY

Figure 4-9. A, mIPSC traces before and during the application of NPY (300 nM). B and C, cumulative fraction plots of the mIPSC interevent interval and amplitude distribution. NPY significantly increased the interevent interval without affecting the amplitude distribution in most neurons (n = 4). The neuron was voltage clamped at a holding potential of –70 mV, with a CsCl-based internal solution in the recording electrode.

Figure 4-9

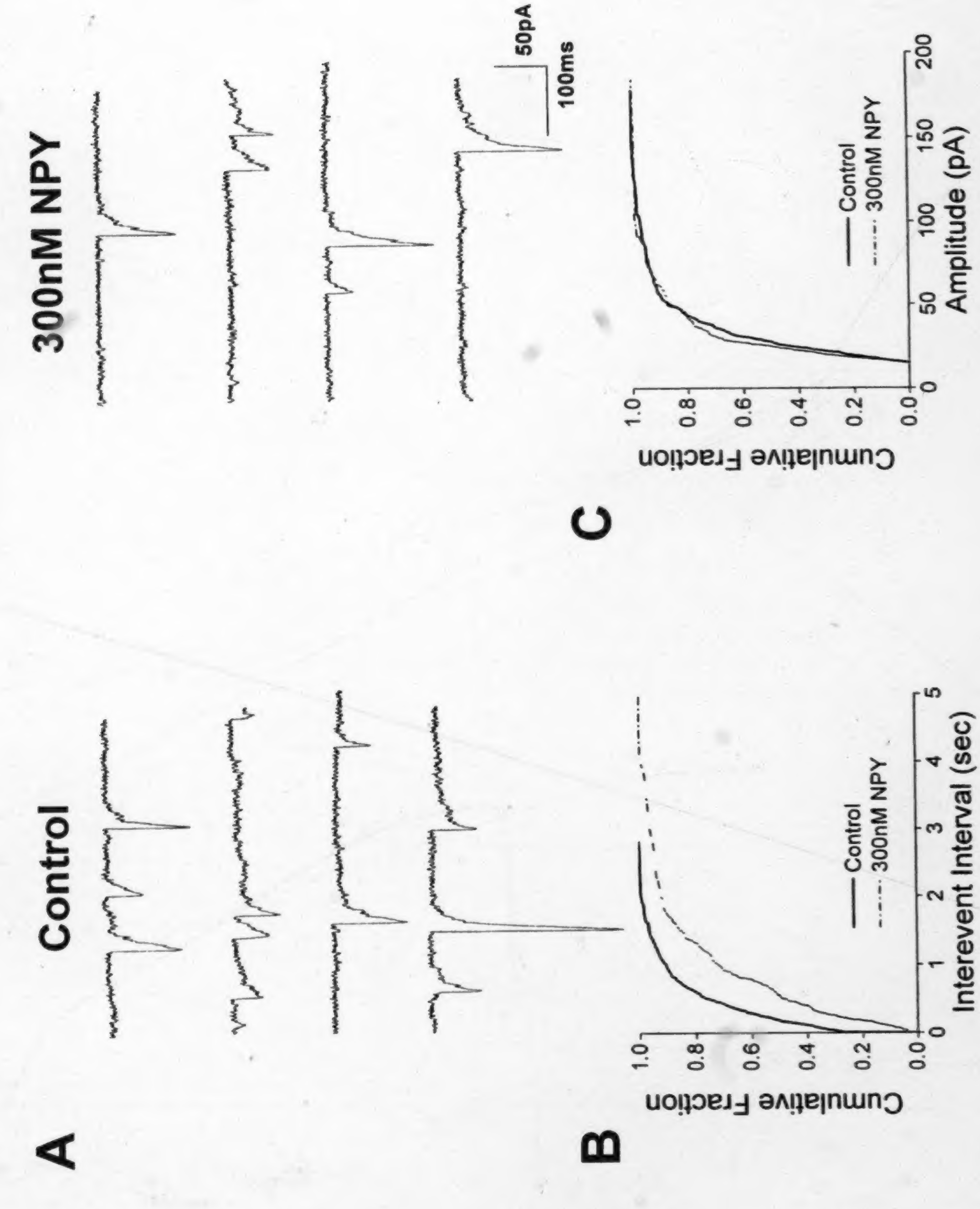

A
Control
300nM NPY
50pA
100ms
B
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Interevent Interval (sec)
Control
300nM NPY
C
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Amplitude (pA)
Control
300nM NPY

Figure 4-10. A, mIPSC traces before and during the application of [F7,P34]NPY (1 μM). B and C, cumulative fraction plots of the mIPSC interevent interval and amplitude distribution. [F7,P34]NPY significantly increased the interevent interval and significantly decreased the amplitude distribution (n = 4), suggesting an action at both pre- and post-synaptic Y1 receptors. The neuron was voltage clamped at a holding potential of −70 mV, with a CsCl-based internal solution in the recording electrode.

Figure 4-10

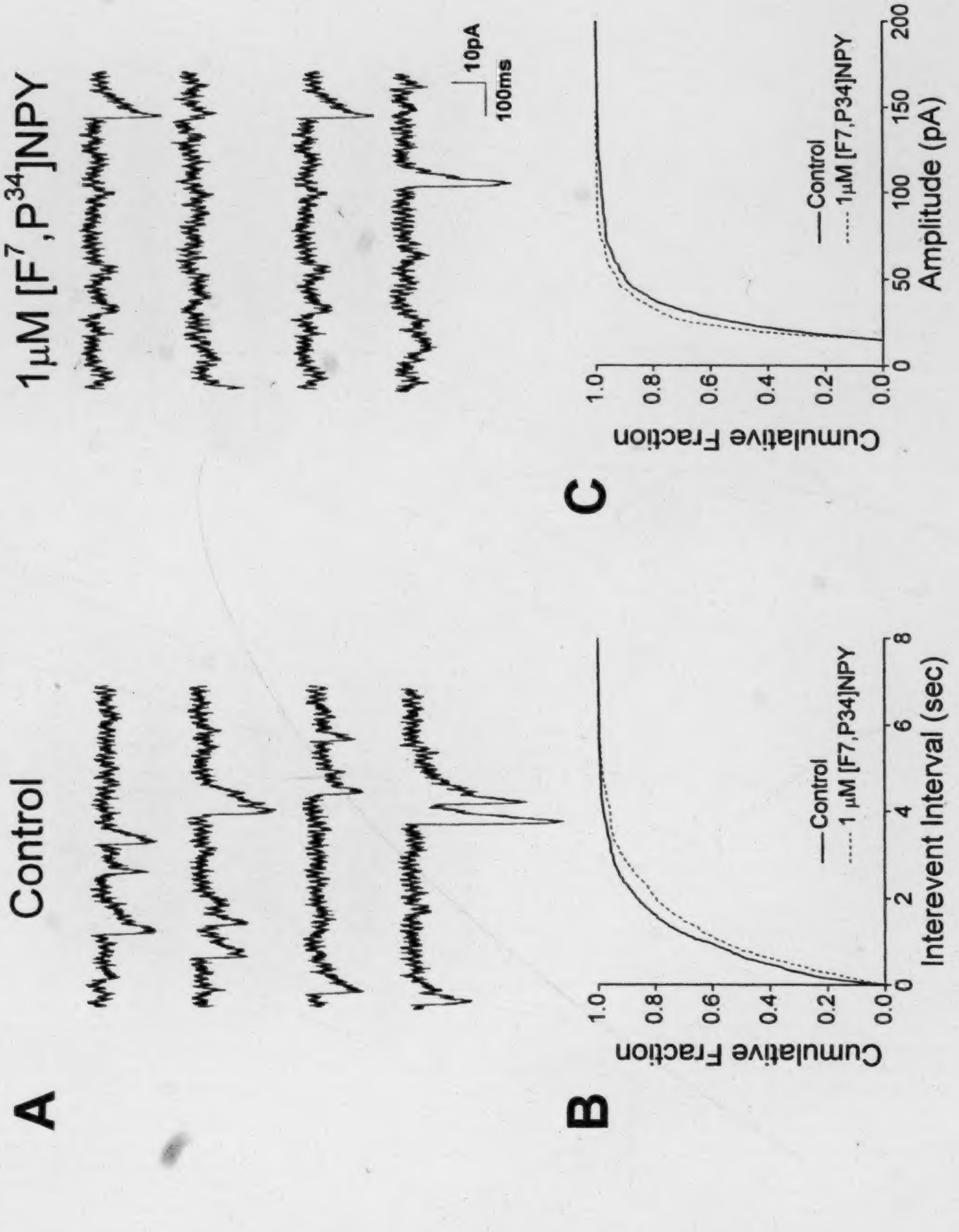

A
Control
1μM [F^7,P^{34}]NPY
10pA
100ms
B
Cumulative Fraction
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
Interevent Interval (sec)
—Control
----- 1 μM [F7,P34]NPY
C
Cumulative Fraction
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
200
Amplitude (pA)
—Control
----- 1μM [F7,P34]NPY

Figure 4-11. NPY has similar actions on GABAergic and glycinergic synaptic transmission in substantia gelatinosa neurons. GABAergic IPSCs were evoked at a holding potential of –70 mV, with a CsCl-based internal solution in the recording electrode, by focal stimulation in the presence of 50 μM AP5, 10 μM CNQX and 1 μM strychnine. A, superimposed, averaged traces (n = 6) of evoked GABAergic IPSC before, during and after application of NPY (300 nM). Glycinergic IPSCs were evoked at a holding potential of –70 mV by focal stimulation in the presence of 50 μM AP5, 10 μM CNQX and 10 μM bicuculline. B, superimposed, averaged traces (n = 6) of evoked glycinergic IPSCs before, during and after application of NPY (300 nM). Note that both GABA- and glycine-mediated IPSCs are similarly affected by NPY. C and D, summary histograms of the effect of NPY on GABAergic (n = 3) and glycinergic (n = 3) IPSCs.

Figure 4-11

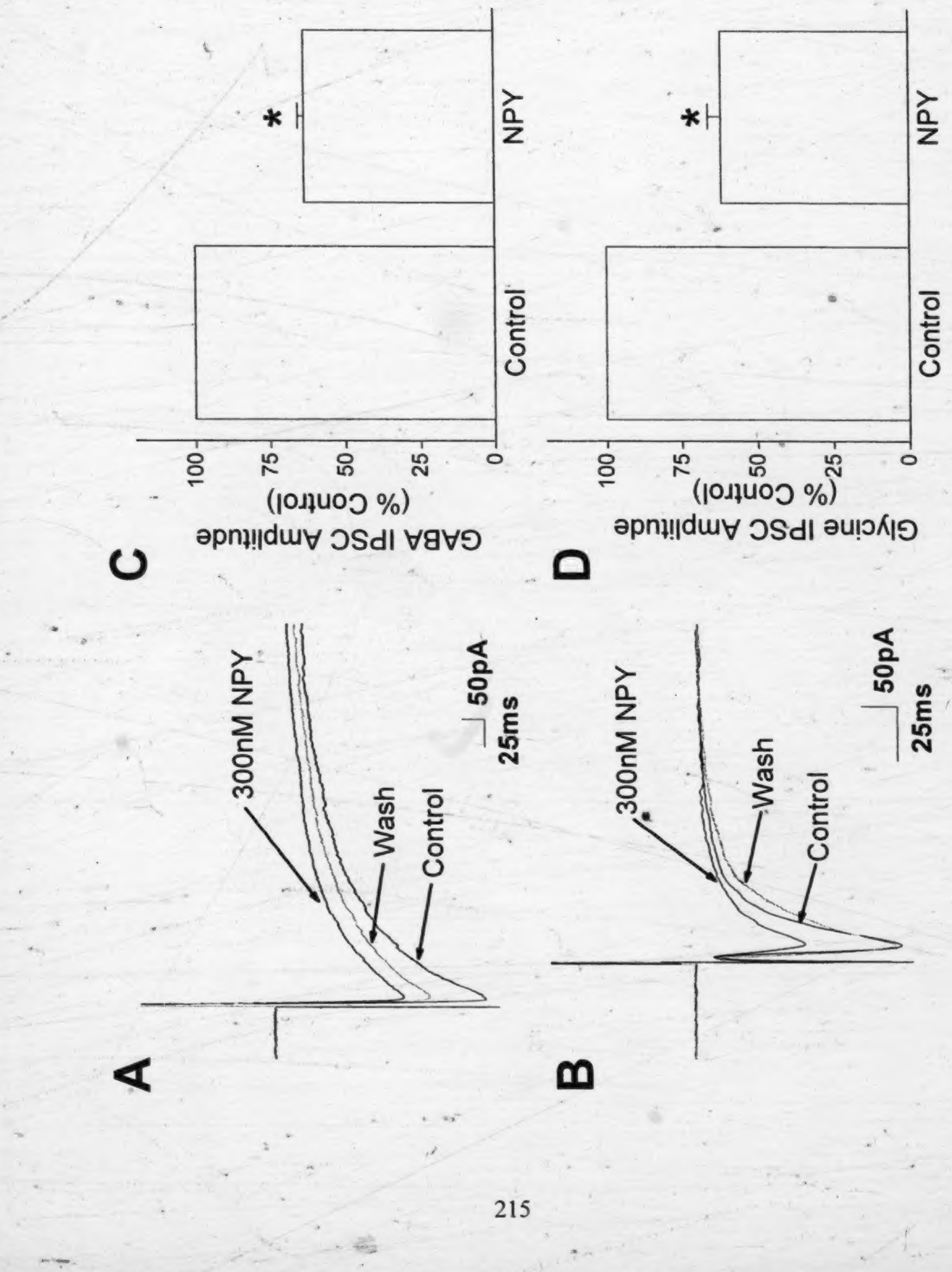

A
300nM NPY
Wash
Control
50pA
25ms
B
300nM NPY
Wash
Control
50pA
25ms
C
GABA IPSC Amplitude (% Control)
0
25
50
75
100
Control
NPY
*
D
Glycine IPSC Amplitude (% Control)
0
25
50
75
100
Control
NPY
*

Figure 4-12. The Y1 receptor antagonist BIBP3226, blocks the action of NPY on evoked IPSCs in substantia gelatinosa neurons. IPSCs were evoked at a holding potential of −70 mV, with a CsCl-based internal solution in the recording electrode. A, superimposed, averaged traces (n = 6) of evoked IPSCs before and during application of NPY (300 nM) in the presence of BIBP3226 (1 μM). Note that BIBP3226 reverses the effect of NPY on the evoked IPSC. B, time course of changes in the amplitude of evoked IPSCs during application of NPY and BIBP3226.

Figure 4-12

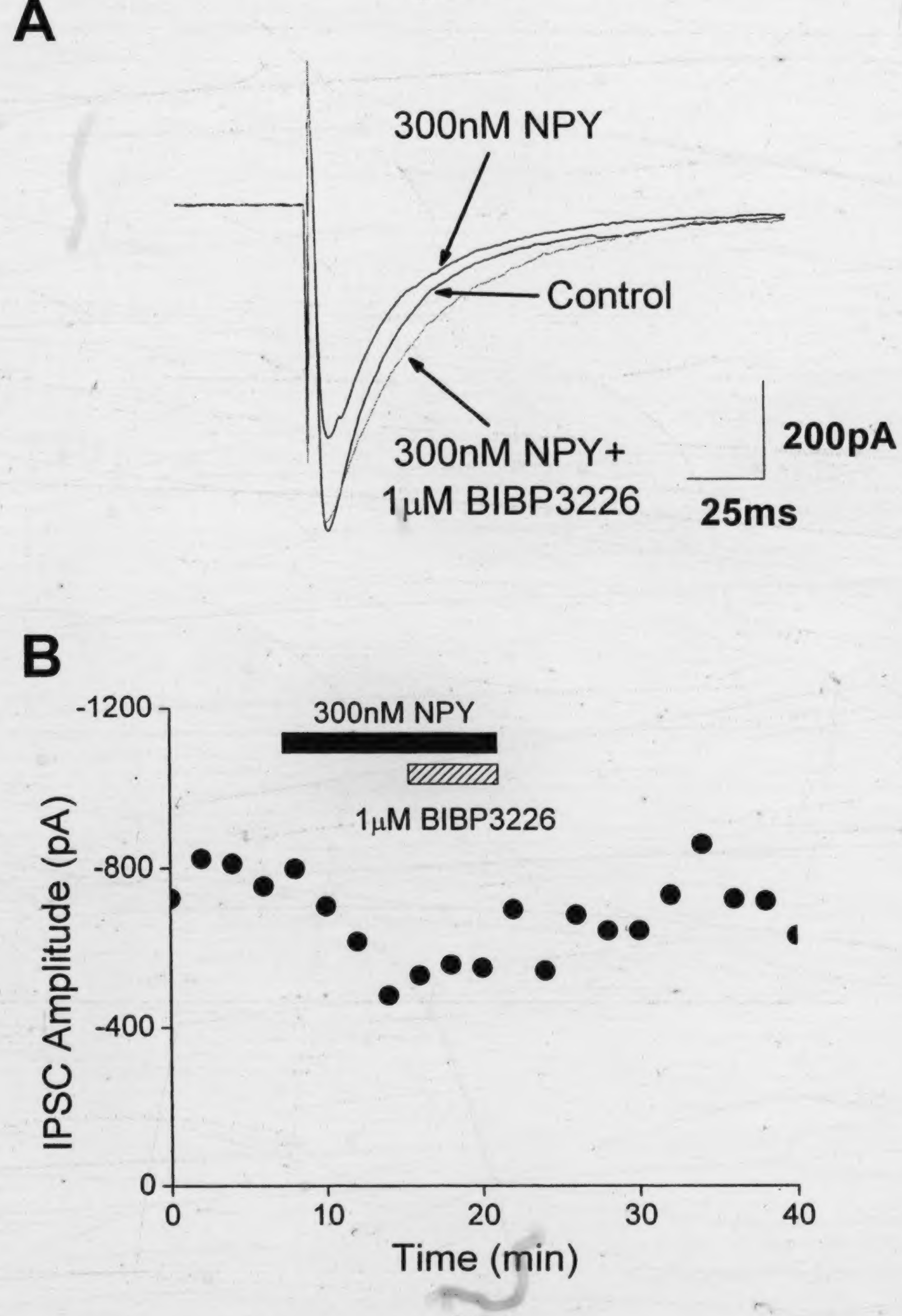

A
300nM NPY
Control
300nM NPY+
1μM BIBP3226
200pA
25ms
B
300nM NPY
1μM BIBP3226
-1200
-800
-400
0
IPSC Amplitude (pA)
0
10
20
30
40
Time (min)

Figure 4-13. The Y2 receptor antagonist BIIE0246, blocks the action of NPY on evoked EPSCs in substantia gelatinosa neurons. EPSCs were evoked at a holding potential of –70 mV, with a CsCl-based internal solution in the recording electrode. A, superimposed, averaged traces (n = 6) of evoked EPSCs before, during and after application of NPY (300 nM). B, in the presence of BIIE0246 (100 nM), the action of NPY on the EPSC is blocked. Note the kinetic changes in the EPSC following application of BIIE0246. C, time course of changes in the amplitude of evoked EPSCs during application of NPY and BIIE0246. Data points shown in panels A and B are numbered for clarity.

Figure 4-13

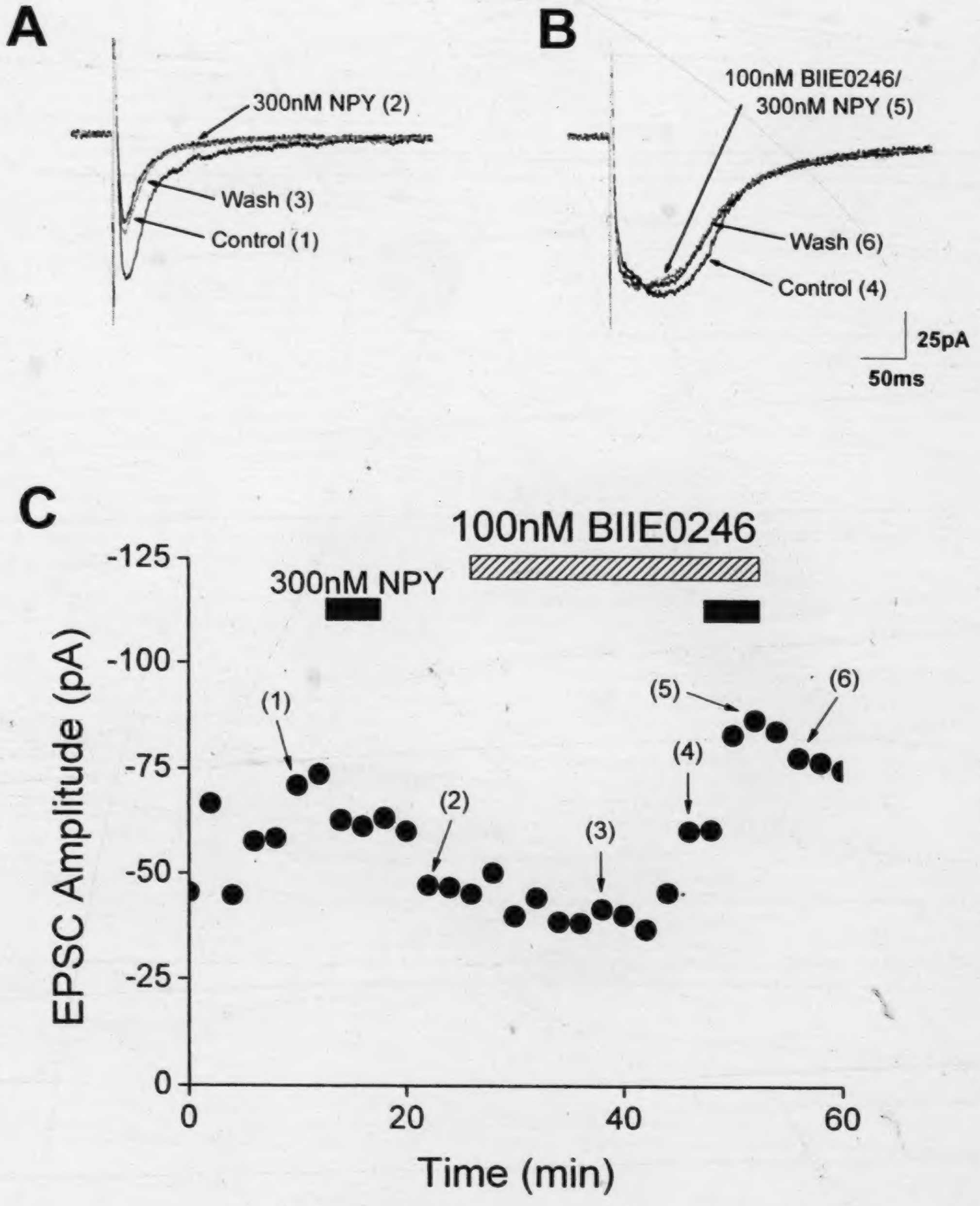

A
300nM NPY (2)
Wash (3)
Control (1)
B
100nM BIIE0246/
300nM NPY (5)
Wash (6)
Control (4)
25pA
50ms
C
100nM BIIE0246
300nM NPY
-125
-100
-75
-50
-25
0
EPSC Amplitude (pA)
(1)
(2)
(3)
(4)
(5)
(6)
0
20
40
60
Time (min)

Figure 4-14. Comparison of the actions of NPY and DAMGO on evoked EPSCs in substantia gelatinosa neurons. EPSCs were evoked by stimulating the dorsal root or dorsal root entry zone in the presence of 10 μM bicuculline and 1 μM strychnine. A, averaged traces (n = 3) of evoked EPSCs before, during and after application of NPY (1 μM). B, averaged traces (n = 3) of evoked EPSCs before, during and after application of DAMGO (1μM) to the same cell. Note that NPY and DAMGO cause a similar reduction in evoked EPSC amplitude. C, time course of the changes in amplitude of evoked EPSCs during application of NPY and DAMGO. Graph in C refers to cell illustrated in A and B. The cell was voltage-clamped at a holding potential of –60 mV with a CsCl-based internal solution in the recording electrode . Presynaptic fibres synapsing onto this cell expressed both NPY and μ-opioid receptors. D, summary histogram illustrating the similar effect of NPY and DAMGO on the evoked EPSC. E, histogram showing the number of cells that responded to NPY (71%; n = 17/24), DAMGO (100%; n = 9/9 cells) and to both agonists (78%; n = 7/9 cells).

Figure 4-14

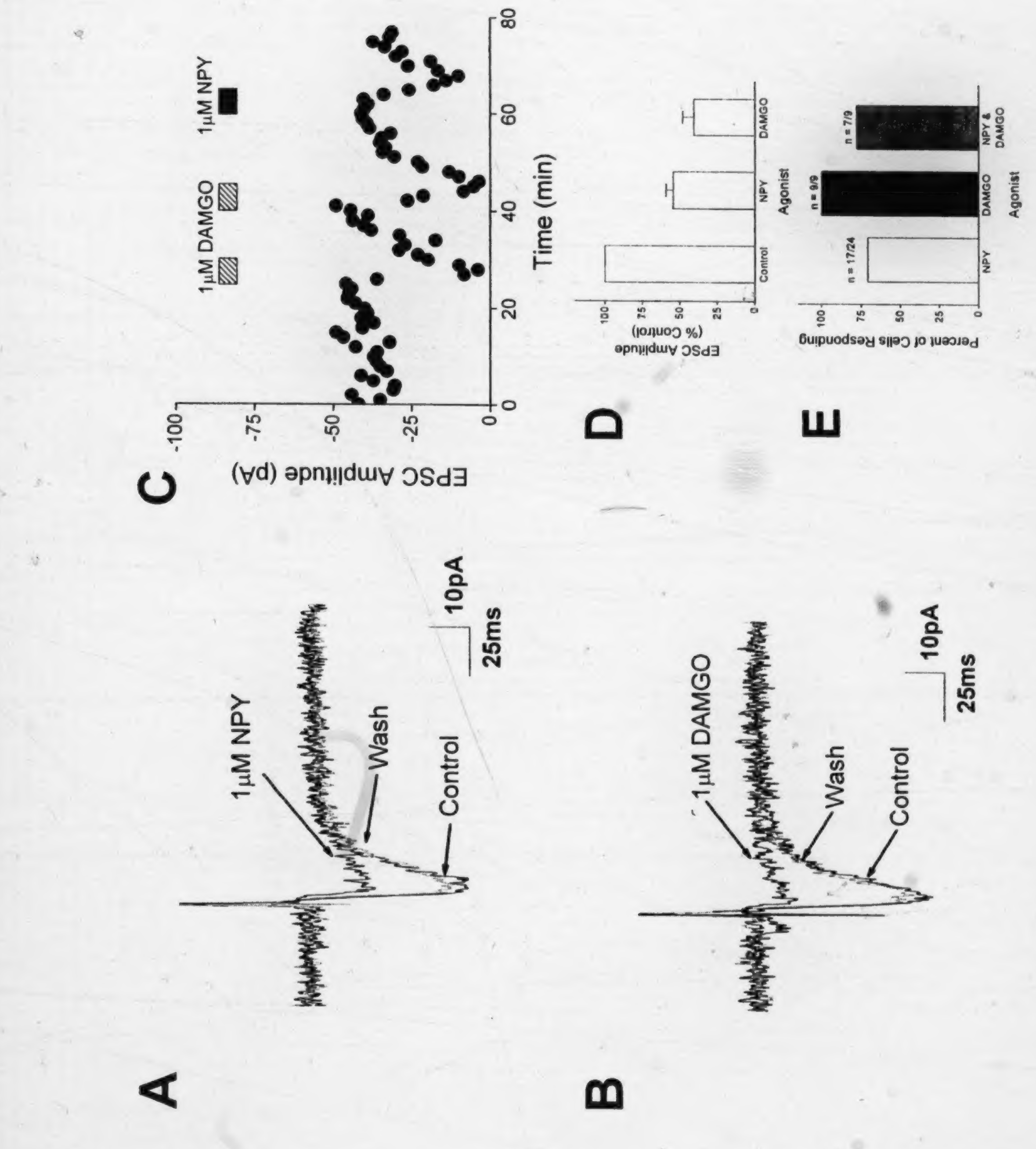
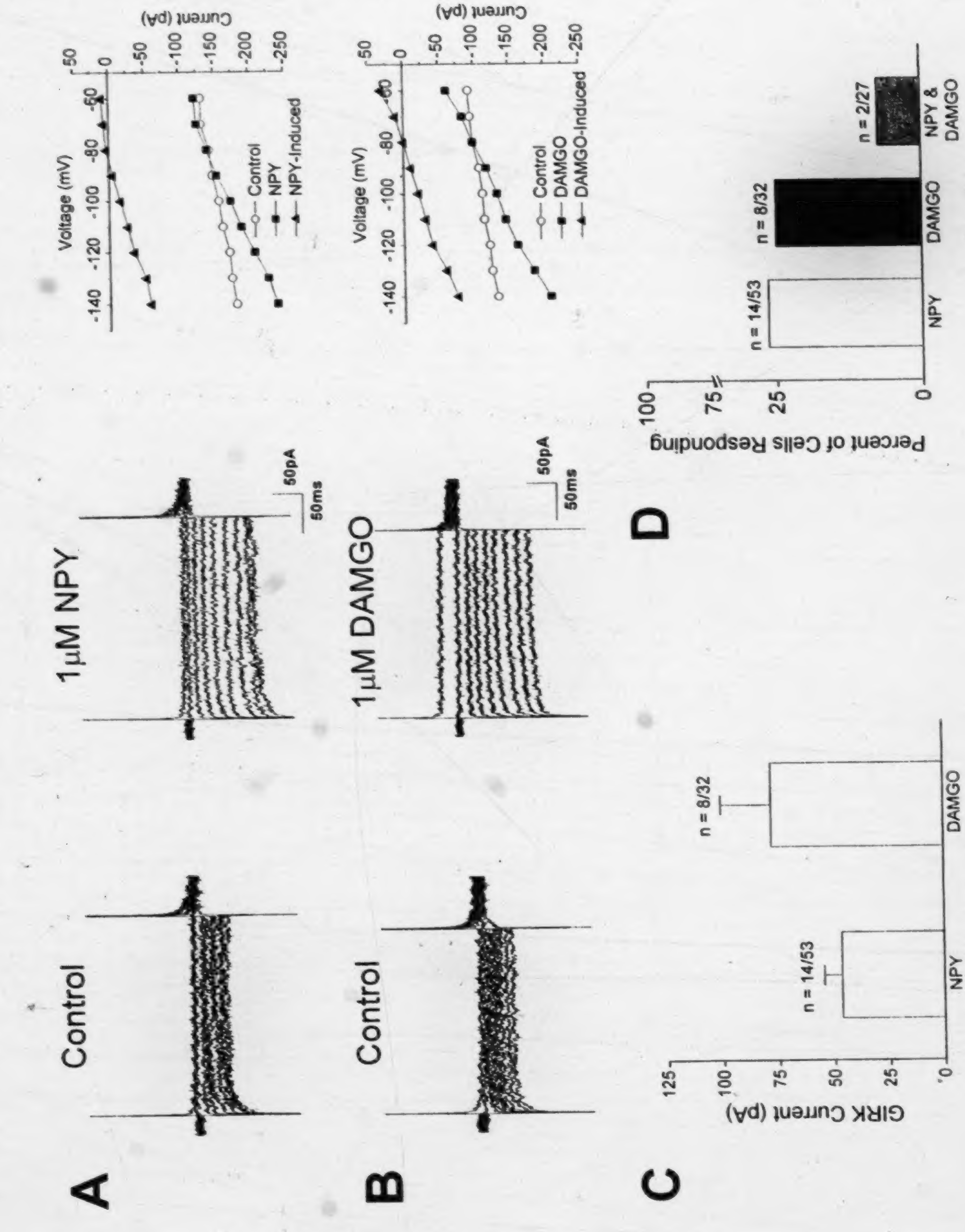

A
1µM NPY
Wash
Control
10pA
25ms
B
1µM DAMGO
Wash
Control
10pA
25ms
C
1µM DAMGO
1µM DAMGO
1µM NPY
EPSC Amplitude (pA)
-100
-75
-50
-25
0
0
20
40
60
80
Time (min)
D
EPSC Amplitude (% Control)
100
75
50
25
0
Control
NPY
DAMGO
Agonist
E
Percent of Cells Responding
100
75
50
25
0
n = 17/24
n = 9/9
n = 7/9
NPY
DAMGO
NPY & DAMGO
Agonist

Figure 4-15


A
Control
1μM NPY
50pA
50ms
Voltage (mV)
-140
-120
-100
-80
-60
Current (pA)
50
0
-50
-100
-150
-200
-250
Control
NPY
NPY-Induced
B
Control
1μM DAMGO
50pA
50ms
Voltage (mV)
Current (pA)
Control
DAMGO
DAMGO-Induced
C
GIRK Current (pA)
125
100
75
50
25
0
n = 14/53
n = 8/32
NPY
DAMGO
Agonist
D
Percent of Cells Responding
100
75
25
0
n = 14/53
n = 8/32
n = 2/27
NPY
DAMGO
NPY & DAMGO
Agonist

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Chapter 5

Morphine-3β-D-Glucuronide Suppresses Inhibitory Synaptic Transmission in Rat Substantia Gelatinosa

Introduction

As mentioned in the General Introduction, initial interest in the actions of morphine-3β-D-glucuronide (M3G) in the dorsal horn arose from the hypothesis that it might act as an ORL_1 receptor agonist. This hypothesis was rejected shortly after initiating studies to examine the effect of M3G as it proved to be incorrect. However, the study was continued and the actions of M3G were compared with μ-opioids and N/OFQ. During the course of these experiments, new findings on the effects of M3G were uncovered.

Morphine is widely used for the management of moderate to severe pain. It is converted by glucuronidation into two major metabolites, M3G and morphine-6β-D-glucuronide (M6G) (Boerner *et al.*, 1975; Christrup, 1997). M6G has high affinity for the μ-opioid receptor (Löser *et al.*, 1996; Pasternak *et al.*, 1987; Paul *et al.*, 1989) and appears to be a more potent opioid agonist than morphine (Frances *et al.*, 1992; Osborne *et al.*, 2000; Pasternak *et al.*, 1987; Paul *et al.*, 1989). In contrast, M3G does not bind to μ-, δ- or κ-opioid receptors (Lambert *et al.*, 1993; Löser *et al.*, 1996; Pasternak *et al.*, 1987) and appears to be devoid of analgesic activity (Pasternak *et al.*, 1987; Yaksh & Harty, 1988). Furthermore, M3G does not interact with NMDA, $GABA_A$ or glycine receptors (Bartlett *et al.*, 1994) and has no effect on membrane conductance or action potential discharge in locus coeruleus neurons (Osborne *et al.*, 2000). Also, M3G does not affect Aβ- or C-fibre-evoked responses in dorsal horn neurons (Hewett *et al.*, 1993; Sullivan *et al.*, 1989). It does, however, produce hyperalgesia and allodynia when administered intrathecally or intracerebroventricularly (Woolf, 1981) and progressively higher doses can cause seizures (Halliday *et al.*, 1999; Smith *et al.*, 1990). These findings are consistent with the suggestion that morphine metabolites may be responsible for the development of hyperalgesia, allodynia and myoclonus during clinical opioid therapy (De Conno *et al.*, 1991; Sjogren *et al.*, 1998).

Therefore, the aim of our revised study of M3G action was to examine its cellular effects on neurons in the rat substantia gelatinosa. Actions of M3G were compared with those of the μ-opioid agonist, DAMGO, and the ORL_1 agonist, nociceptin/orphanin FQ (N/OFQ). Although it is established that M3G does not interact with μ-, δ- or κ-receptors, we sought to examine possible interactions with other mechanisms within the dorsal horn, including the more recently defined ORL_1 receptor (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). A version of this chapter has been published in the *Journal of Pharmacology and Experimental Therapeutics* (Moran & Smith, 2002).

Methods

The methods for recording from substantia gelatinosa neurons were identical to those described in Chapter 2, except the slices were allowed to equilibrate to room temperature (~22°C) for 1 h prior to recording. These experiments were made using a K^+-gluconate-based internal solution and the holding potential was –60 or –70 mV for recording EPSCs and 0 mV for recording IPSCs.

Results

M3G Does Not Affect Evoked Excitatory Postsynaptic Currents

Whole-cell patch-clamp recordings were obtained from substantia gelatinosa neurons from slices maintained *in vitro* for up to 10 h and stable recordings were made from individual neurons for up to 3 h.

In the presence of bicuculline (10 μM) and strychnine (1 μM), stimulation of the dorsal root or dorsal root entry zone generated EPSCs in substantia gelatinosa neurons at a holding potential of –60 or –70 mV. Superfusion of M3G (1-100 μM) had no significant effect on EPSC amplitude in any of the 19 cells tested. By contrast, and confirming previous reports (Glaum *et al.*, 1994; Kohno *et al.*, 1999), the μ-opioid agonist DAMGO (1 μM) reduced EPSC amplitude by an average of 46.9 ± 4.82% (n = 19/25 cells tested). Similarly, the ORL_1 agonist N/OFQ (1 μM) reduced EPSC amplitude by 39.6 ± 7.10% (n = 5/6 cells tested), which confirms previous findings (Lai *et al.*, 1997; Liebel *et al.*, 1997). Sample data records are shown in Figure 5-1. In Figure 5-1A, the EPSC is unaffected by M3G but is suppressed by N/OFQ. In Figure 5-1B, M3G is again ineffective but the EPSC is suppressed by DAMGO. Time courses of the effects of these drugs are shown in Figure 5-1C and 5-1D. The histogram in Figure 5-1E summarizes the effects of M3G, DAMGO and N/OFQ on evoked EPSCs.

M3G Does Not Interact with ORL_1 or μ-opioid Receptors in Substantia Gelatinosa Neurons

Because M3G had no noticeable effect on excitatory transmission at synapses where N/OFQ and DAMGO suppressed transmission, it is unlikely to act as an ORL_1 or μ-opioid agonist. M3G was also devoid of antagonist activity at these receptors. Superfusion of M3G (1 or 10 μM) did not occlude the effects of N/OFQ. In the presence of M3G, superfusion of N/OFQ (1 μM) reduced EPSC amplitude by 41.8 ± 7.84% ($n = 3$; Figure 5-2A), which is similar to the actions of N/OFQ by itself on EPSC amplitude ($p > 0.85$, t-test; compare with Figure 5-1A). In a similar series of experiments, superfusion of M3G (1 or 10 μM) did not occlude the actions of DAMGO. In the presence of M3G, superfusion of DAMGO reduced EPSC amplitude by 40.9 ± 4.30% ($n = 3$; Figure 5-2B), which is similar to the actions of DAMGO alone ($p > 0.85$, t-test; compare with Figure 5-1B). Time courses of these drug effects on EPSC amplitude are shown in Figure 5-2C and 5-2D. These observations are consistent with binding studies that show M3G does not bind to μ-opioid receptors (Lambert *et al.*, 1993; Löser *et al.*, 1996; Pasternak *et al.*, 1987).

Comparison of Postsynaptic Actions of M3G, Nociceptin/Orphanin FQ and DAMGO

Membrane conductance measured from a voltage-ramp protocol was unaffected by M3G (1 μM, $n = 11$ or 100 μM, $n = 5$). Figure 5-3A shows the lack of effect of 100 μM M3G on currents evoked by a voltage ramp from −140 to 0 mV. In the same cell (Figure 5-3B), 1 μM DAMGO increased conductance at negative voltages, reflecting its activation of an inwardly-rectifying conductance (Grudt & Williams, 1994; Schneider *et al.*, 1998). M3G (1 μM) also had no effect on excitability (n = 5), as evaluated by the frequency of action potential discharge in response to depolarizing current pulses (data not shown). These findings are similar to those of Osborne *et al.* (2000) who found no effect of M3G on membrane conductance or action potential firing in locus coeruleus neurons.

In contrast to the lack of effect of M3G on membrane conductance, N/OFQ (1 μM; 8/14 cells) activated an inwardly-rectifying conductance which was reflected by a 49.0 ± 5.98 pA increase in current at –140 mV (Figure 5-3C). This was very similar to the findings in medullary dorsal horn (Jennings, 2001). The reversal potential for the N/OFQ-induced current of –97.0 ± 2.51 mV in 2.5 mM $[K^+]_o$ (n = 8) was shifted to –75.9 ± 4.28 mV in 6.5 mM $[K^+]_o$ (n = 3), consistent with the activation of a K^+ conductance. These values are similar to the calculated E_K (-99.8 mV in 2.5 mM $[K^+]_o$ and -75.7 mV in 6.5 mM $[K^+]_o$). DAMGO (1 μM) also activated an inwardly-rectifying K^+ current of 60.5 pA ± 10.0 pA (n = 5/12 cells tested) at –140 mV (Figure 5-3D), which confirms previous reports (Grudt & Williams, 1994; Schneider *et al.*, 1998).

Actions of M3G on Evoked and Miniature Inhibitory Postsynaptic Currents

In the presence of the glutamate receptor antagonists, AP5 (50 μM) and CNQX (10 μM), focal stimulation generated IPSCs in substantia gelatinosa neurons at a holding potential of 0 mV. In contrast to its lack of effect on evoked EPSCs, M3G produced a concentration-dependent decrease in the amplitude of the evoked IPSC (Figure 5-4A).

This effect was seen in all cells tested with M3G. Sample data records are illustrated in Figure 5-4B.

Because HPLC analysis indicated that our M3G contained ~0.28% morphine, it was possible that the effect on the IPSC was caused by the small amount of morphine in our sample. Therefore, the effect of 100 μM M3G was compared with that of 300 nM morphine. This low concentration of morphine caused a negligible reduction in IPSC amplitude (9.73 ± 6.19%; $n = 3$; Figure 5-4B and 5-4C). Figure 5-4C shows the time course of the effect of 100 μM M3G and 300 nM morphine on IPSC amplitude. It was also possible that our sample of M3G was contaminated with a small amount of M6G, which is a potent μ-agonist (Osborne *et al.*, 2000). To test for this possibility, effects of 100 μM M3G on the IPSC were studied in the presence of 100 μM naloxone. Data records are shown in Figure 5-4D and the time course of the effect of M3G in the presence of naloxone is shown in 5-4E. Because the effect of M3G was unchanged, the actions of M3G did not reflect contamination of the sample by μ-agonists. Moreover, they confirmed that the action of M3G was not mediated via μ-opioid receptors and exclude possible interactions with δ- and κ-receptors.

To characterize further the action of M3G on inhibitory synaptic transmission, we examined the effect of 100 μM M3G on TTX-insensitive mIPSCs. M3G (100 μM) reduced the frequency ($n = 4/4$ cells tested; $p < 0.05$, Kolmogorov-Smirnov test; Figure 5-5A-C), but had no effect on the amplitude of the mIPSCs ($n = 4/4$ cells tested; $p > 0.05$, Kolmogorov-Smirnov test; Figure 5-5C). This preferential effect on mIPSC frequency rather than amplitude suggested that M3G acted presynaptically. Additional evidence for a presynaptic site of action of M3G was obtained from paired-pulse experiments. Two identical stimuli separated by an inter-stimulus interval (50-100 ms) resulted in paired-pulse facilitation of the evoked IPSC. The mean ratio of the amplitude of the paired IPSCs was 1.04 ± 0.09 ($IPSC_2/IPSC_1$; $n = 4$). In 4 out of 6 cells, superfusion of M3G (100 μM) produced an increase in the mean ratio of $IPSC_2/IPSC_1$ to 1.67 ± 0.45 ($n = 4$). This reflected suppression of the evoked IPSC and a 29.9 ± 8.95% increase in the paired-pulse ratio ($p < 0.005$, paired t-test, $n = 4$). Sample data records are shown in Figure 5-6A. In Figure 5-6B, the IPSCs have been normalized to the amplitude of $IPSC_1$ to better illustrate the change in the paired-pulse ratio. Figure 5-6C is a summary histogram of the effect of 100μM M3G on the paired-pulse ratio.

Evoked IPSCs in the spinal cord comprise GABAergic and glycinergic components. To examine whether M3G selectively affected one of these components, we examined its effect on evoked GABAergic IPSCs in the presence of AP5, CNQX and strychnine (1 μM) and glycinergic IPSCs in the presence of AP5, CNQX and bicuculline (10 μM). Both components of the IPSC were similarly affected. Thus, in 4/4 cells tested, M3G (100 μM) suppressed the GABAergic IPSC by 48.7 ± 12.8% ($p < 0.05$) and in 4/5 cells tested, it suppressed the glycinergic IPSC by 39.2 ± 4.80% ($p < 0.05$). Sample data records for GABAergic and glycinergic IPSCs are shown in Figure 5-7A and 5-7B, respectively. Summary histograms are shown in Figure 5-7C and 5-7D.

Actions of Nociceptin/Orphanin FQ and DAMGO on Evoked Inhibitory Postsynaptic Currents

Recently, N/OFQ has been reported to selectively suppress glutamatergic synaptic inputs in the spinal cord dorsal horn (Ahmadi *et al.*, 2001; Zeilhofer *et al.*, 2000), but to have no effect on inhibitory synaptic currents. We observed a similar lack of effect of N/OFQ (1 μM) on evoked IPSCs. N/OFQ reduced the amplitude of evoked IPSCs by only 5.88 ± 0.75% ($p > 0.05$, $n = 5/5$ cells tested; Figure 5-8A). The actions of N/OFQ on synaptic transmission in the substantia gelatinosa are the reverse of M3G, which inhibits IPSCs, but fails to affect EPSCs. Furthermore, DAMGO (1 μM) reduced IPSC amplitude by 49.6 ± 10.8%, ($n = 6/10$ cells tested, $p < 0.05$; Figure 5-8B), confirming the findings of Grudt & Henderson (1998), but contradicting those of Kohno *et al.* (1999) who found that DAMGO did not inhibit IPSCs in rat lumbar dorsal horn. Figure 5-8C shows the time course of the effect of DAMGO on IPSC amplitude. Figure 5-8D is a summary histogram that compares the effect of DAMGO, M3G and N/OFQ on IPSC amplitude.

Discussion

In this study, the cellular effects of M3G were compared with those of the ORL_1 agonist, N/OFQ, and the μ-opioid agonist, DAMGO, in substantia gelatinosa neurons of rat lumbar spinal cord. M3G had little or no effect on excitatory synaptic transmission at synapses where ORL_1 or μ-opioid agonists were effective. M3G also failed to affect postsynaptic membrane conductance or excitability, whereas both N/OFQ and DAMGO activated an inwardly-rectifying K^+ conductance. Moreover, suppression of excitatory synaptic responses by N/OFQ or DAMGO was not antagonized by M3G. M3G is therefore neither an agonist nor an antagonist at ORL_1 or μ-opioid receptors. However, M3G produced a naloxone-insensitive, concentration-dependent suppression on inhibitory synaptic transmission. The GABAergic and glycinergic components of the IPSC were similarly affected. Analysis of TTX-insensitive mIPSCs indicated that this action of M3G was presynaptic. This finding was supported by paired-pulse experiments where M3G produced an increase in paired-pulse facilitation.

The lack of effect of M3G (1-100 μM) on EPSCs is consistent with binding studies (Bartlett *et al.*, 1994) and neurochemical assays that showed M3G has no affinity for the NMDA receptor and does not affect the release of glutamic acid from synaptosomes (Bartlett & Smith, 1996). Moreover, intrathecal M3G has no effect on C-fibre-evoked responses in the superficial dorsal horn (Hewett *et al.*, 1993; Sullivan *et al.*, 1989)

The failure of M3G to antagonize the effects of DAMGO and the lack of effect of M3G on evoked EPSCs is consistent with receptor binding studies which show M3G has little or no affinity for the μ-opioid receptor (Löser *et al.*, 1996; Pasternak *et al.*, 1987). In addition, our findings agree with a previous electrophysiological study (Hewett *et al.*, 1993), which indicates M3G does not antagonize the anti-nociceptive actions of intrathecal morphine. Moreover, lack of antagonism of the actions of N/OFQ suggests that M3G also does not interact with the ORL_1 receptor in rat substantia gelatinosa.

By contrast, with its lack of effect on excitatory synaptic transmission, M3G produced a concentration-dependent suppression on inhibitory synaptic transmission. At a concentration of 100 μM, M3G reduced the amplitude of evoked IPSCs by approximately 45%. In the presence of TTX, M3G reduced the mIPSC frequency without affecting the amplitude distribution in all cells tested, suggesting the effect of M3G involved a presynaptic mechanism. If M3G had exerted an effect on postsynaptic $GABA_A$ or glycine receptors, a change in mIPSC amplitude would likely have been observed. Similarly, a postsynaptic action of M3G would not account for the observed increase in the paired-pulse ratio. The effect on inhibitory transmission was not caused by morphine contamination of our sample of M3G because a concentration of morphine (300 nM), equivalent to the amount of contamination had no effect. Involvement of potential μ-agonist contaminants (morphine and M6G) was also ruled out by the lack of effect of naloxone on M3G-induced suppression of IPSCs.

Because intrathecally administered $GABA_A$ and glycine receptor antagonists (Beyer *et al.*, 1985; Kaneko & Hammond, 1997; Yaksh *et al.*, 1986; Zhang *et al.*, 2001) have pro-nociceptive actions similar to M3G, selective suppression of inhibitory synaptic transmission by M3G may explain its allodynic and hyperalgesic effects. It may also explain the allodynia, hyperalgesia and myoclonus observed following administration of high-dose morphine in humans (De Conno *et al.*, 1991; Heger *et al.*, 1999; Sjogren *et al.*, 1993; Sjogren *et al.*, 1994; Sjogren *et al.*, 1998). Our observed effects may be especially relevant to palliative care situations, where heroic doses of morphine (up to 20 g/day) are required to produce analgesia in tolerant individuals (Hagen & Swanson, 1997; Sjogren *et al.*, 1998). In humans, intrathecal injection of 1100 mg of morphine results in an accumulation of ~3 μM M3G in the CSF (Goucke *et al.*, 1994). By extrapolation, the concentration of M3G in the CSF of a palliative care patient who had received 20 g of morphine within a day would approach 60 μM (Hagen & Swanson, 1997; Sjogren *et al.*, 1998). This falls within the range of concentrations tested in the present study. Thus, suppression of GABA- and glycine-mediated synaptic transmission by M3G may explain the development of allodynia, hyperalgesia, seizures and myoclonus that occur with high-dose opioid administration and may dictate the limiting dose of morphine that can be administered.

While it is well established that DAMGO suppresses EPSCs in substantia gelatinosa neurons (Glaum *et al.*, 1994; Kohno *et al.*, 1999), its effect on IPSCs is controversial. In the substantia gelatinosa of the lumbar spinal cord, DAMGO was reported to not affect inhibitory synaptic transmission (Kohno *et al.*, 1999), whereas in trigeminal nucleus pars caudalis, DAMGO suppressed GABAergic and glycinergic IPSCs (Grudt & Henderson, 1998). We found that DAMGO suppressed IPSCs in lumbar substantia gelatinosa, which supports the findings of Grudt & Henderson (1998). One reason for the disparate findings may be the temperature at which the various studies were performed. The work of Kohno *et al.* (1999) was done at 37°C, while our work, and that of Grudt & Henderson (1998) was done at lower temperatures (24°C or 30°C). If there is an increased safety factor for inhibitory synaptic transmission at higher temperatures, this may explain the insensitivity of IPSCs to DAMGO that was noted by Kohno *et al.* (1999). We also corroborated previous findings that N/OFQ selectively suppresses EPSCs in the substantia gelatinosa (Ahmadi *et al.*, 2001; Lai *et al.*, 1997; Liebel *et al.*, 1997; Zeilhofer *et al.*, 2000).

Because actions of M3G at μ-, δ- and κ-opioid and ORL$_1$ receptors (Löser *et al.*, 1996; Pasternak *et al.*, 1987; Sullivan *et al.*, 1989) have now been excluded, the target through which M3G exerts its effect remains to be determined. It is noteworthy that the selective presynaptic effect of M3G on IPSCs is similar to that of the recently identified neuropeptide, nocistatin. Nocistatin, like M3G, selectively suppresses IPSCs in the dorsal horn via a presynaptic mechanism (Zeilhofer *et al.*, 2000) and also has pro-nociceptive actions in behavioural tests (Ahmadi *et al.*, 2001; Xu *et al.*, 1999; Zeilhofer *et al.*, 2000). It is therefore possible that M3G interacts with the nocistatin receptor, but until the nocistatin receptor is better characterized and antagonists are developed, this possibility remains to be investigated.

Figure 5-1

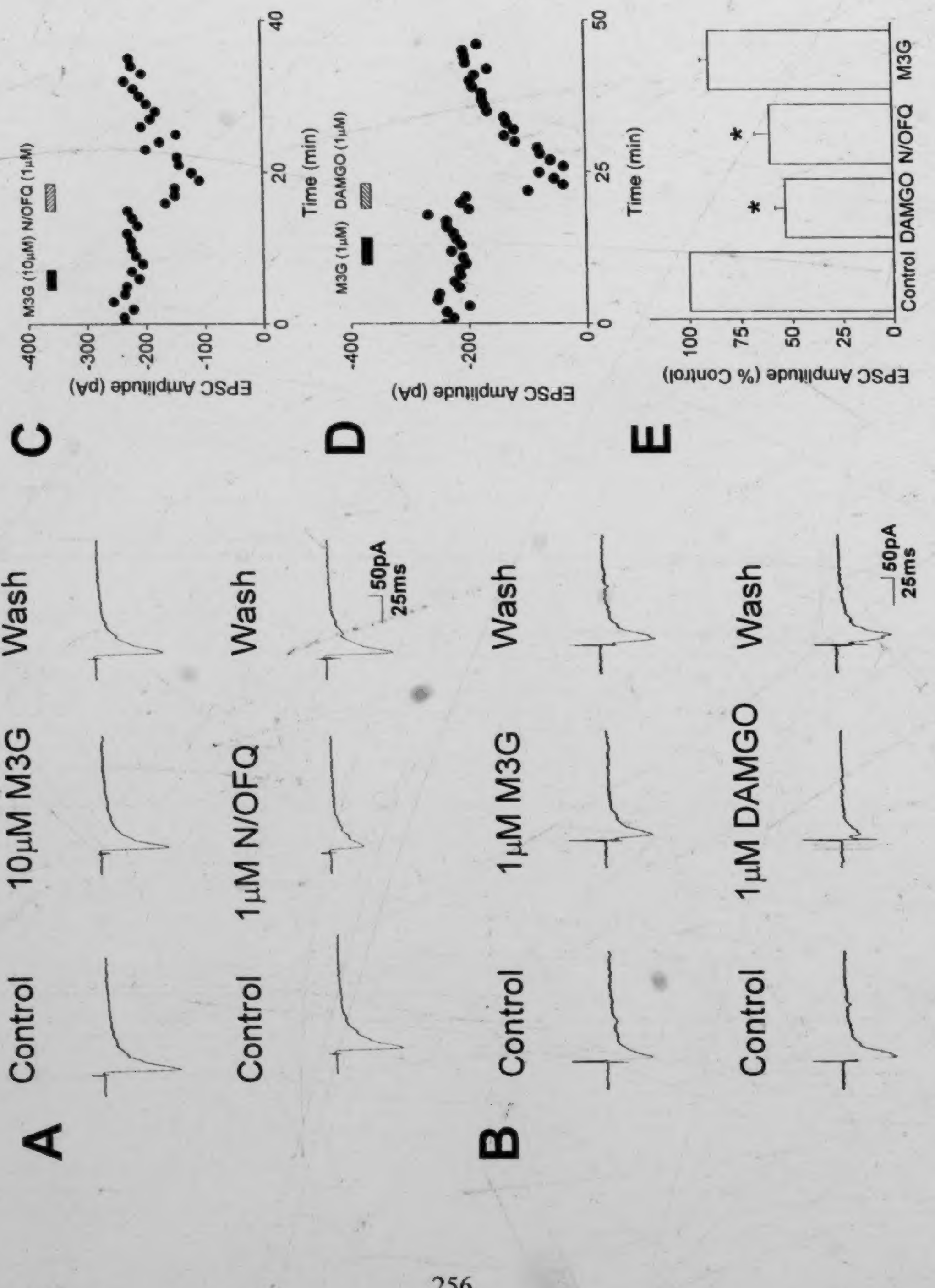

A
Control
10μM M3G
Wash
Control
1μM N/OFQ
Wash
50pA
25ms
B
Control
1μM M3G
Wash
Control
1μM DAMGO
Wash
50pA
25ms
C
M3G (10μM) N/OFQ (1μM)
EPSC Amplitude (pA)
-400
-300
-200
-100
0
0
20
40
Time (min)
D
M3G (1μM) DAMGO (1μM)
EPSC Amplitude (pA)
-400
-200
0
0
25
50
Time (min)
E
EPSC Amplitude (% Control)
0
25
50
75
100
*
*
Control
DAMGO
N/OFQ
M3G

Figure 5-2. M3G is not an antagonist at ORL_1 or μ-opioid receptors in substantia gelatinosa neurons. EPSCs were evoked by stimulating the dorsal root in the presence of 10 μM bicuculline and 1 μM strychnine. A and B, averaged traces (n = 3) of evoked EPSCs from a holding potential of –60 mV (K^+-gluconate-based internal solution in the patch pipette). M3G (10 μM) does not affect N/OFQ or DAMGO-induced suppression of the evoked EPSC. C and D, time course of changes in the amplitude of evoked EPSCs. Graph in C refers to cell illustrated in A and graph in D refers to that in B.

Figure 5-2

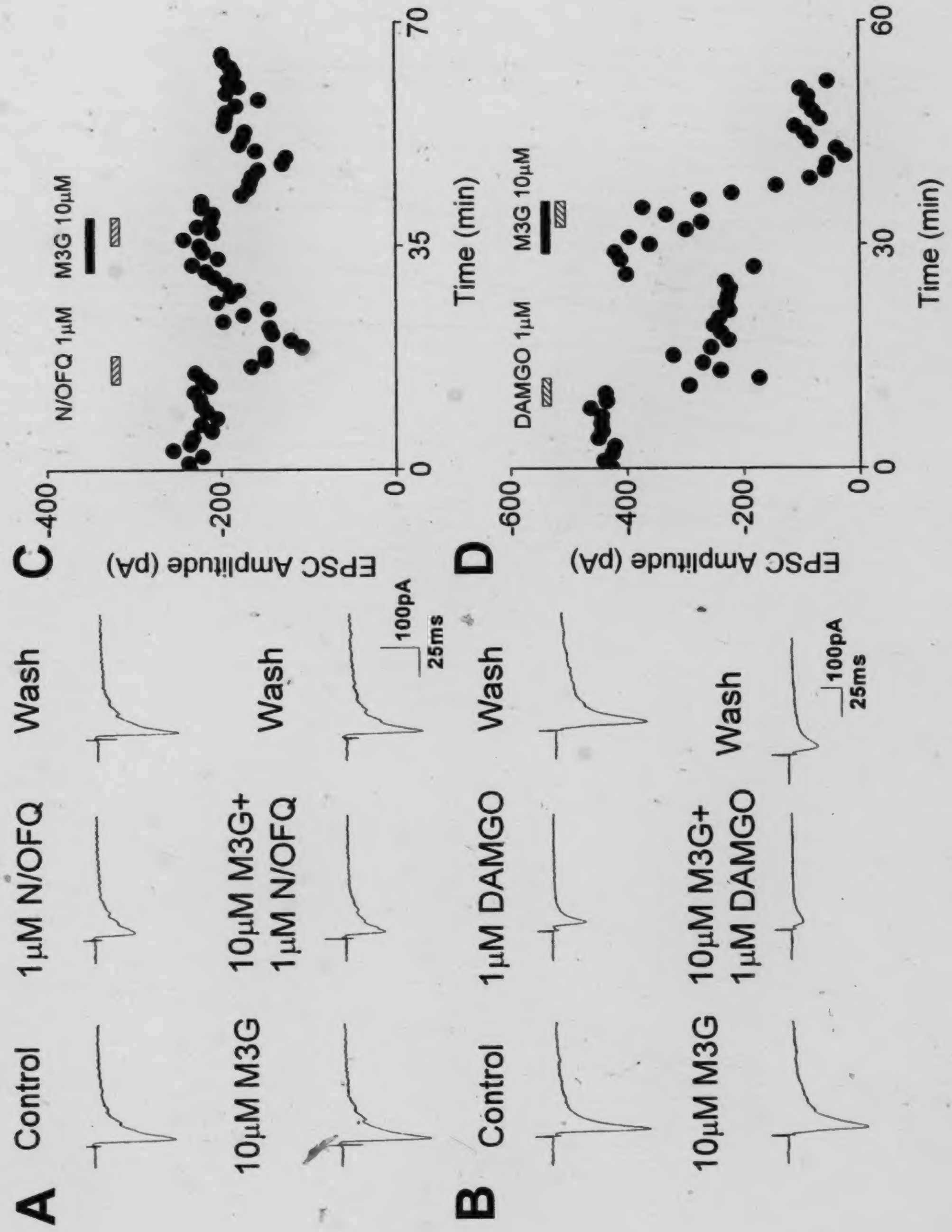

A
Control
1µM N/OFQ
Wash
10µM M3G
10µM M3G+
1µM N/OFQ
Wash
100pA
25ms
B
Control
1µM DAMGO
Wash
10µM M3G
10µM M3G+
1µM DAMGO
Wash
100pA
25ms
C
N/OFQ 1µM
M3G 10µM
EPSC Amplitude (pA)
-400
-200
0
0
35
70
Time (min)
D
DAMGO 1µM
M3G 10µM
EPSC Amplitude (pA)
-600
-400
-200
0
0
30
60
Time (min)

Figure 5-3. N/OFQ and DAMGO activate an inwardly-rectifying K$^+$ conductance whereas M3G does not affect post-synaptic membrane properties in substantia gelatinosa neurons. A, a voltage ramp (1.5 s) was applied to a substantia gelatinosa neuron from –140 to 0 mV. The holding potential was –70 mV (K$^+$-gluconate-based internal solution in the patch pipette). The membrane currents were recorded before and during application of M3G (100 μM). B, DAMGO (1 μM) was applied to the cell in A. Note the increased conductance at negative voltages, reflecting its activation of an inwardly-rectifying conductance. C, an inwardly-rectifying K$^+$ conductance activated by N/OFQ (1 μM) in a substantia gelatinosa neuron. Voltage command steps of 250 ms duration were made in 10 mV incremental steps from –40 to –140 mV before (left panel) and during (centre panel) superfusion of 1 μM N/OFQ. The holding potential was –60 mV (K$^+$-gluconate-based internal solution in the patch pipette). In the right panel, a current-voltage relationship for the control current, the current seen in N/OFQ and the N/OFQ-induced current obtained by subtraction were plotted from the current traces at left. The N/OFQ-induced current exhibits clear inward-rectification. D, an inwardly-rectifying K$^+$ conductance activated by DAMGO (1 μM) in a substantia gelatinosa neuron. Voltage command steps of 250 ms duration were made in 10 mV incremental steps from –50 to –140 mV before (left panel) and during (centre panel) superfusion of 1 μM DAMGO. The holding potential was –70 mV (K$^+$-gluconate-based internal solution in the patch pipette). The right panel shows a current-voltage relationship for the control current, the current seen in DAMGO and the DAMGO-induced current obtained by subtraction and plotted from the current traces at left.

Figure 5-3

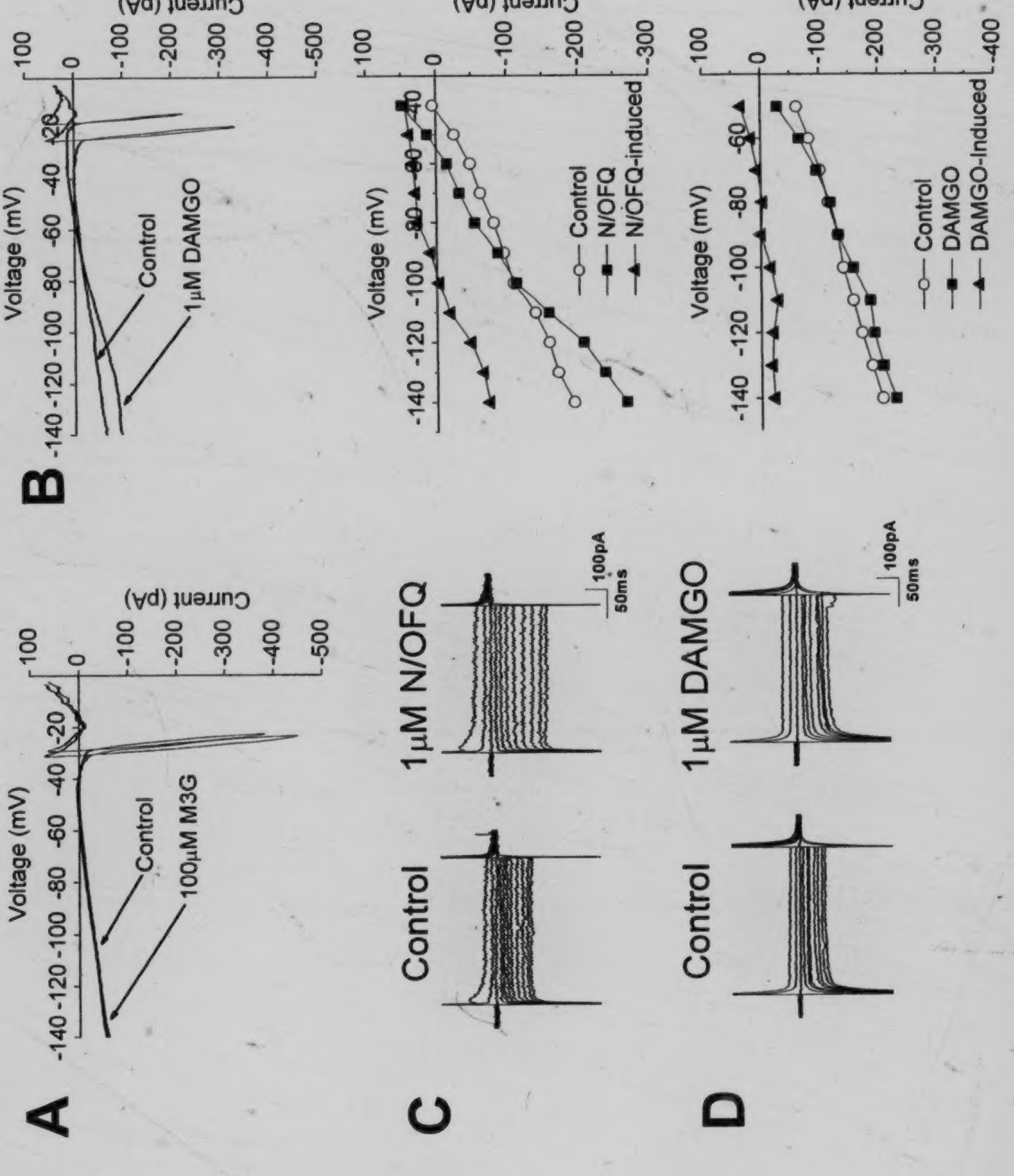

A
Voltage (mV)
-140 -120 -100 -80 -60 -40 -20
Current (pA)
100 0 -100 -200 -300 -400 -500
Control
100μM M3G
B
Voltage (mV)
-140 -120 -100 -80 -60 -40 -20
Current (pA)
100 0 -100 -200 -300 -400 -500
Control
1μM DAMGO
C
Control
1μM N/OFQ
100pA
50ms
Voltage (mV)
-140 -120 -100 -80 -60 -40
Current (pA)
100 0 -100 -200 -300
Control
N/OFQ
N/OFQ-induced
D
Control
1μM DAMGO
100pA
50ms
Voltage (mV)
-140 -120 -100 -80 -60
Current (pA)
100 0 -100 -200 -300 -400
Control
DAMGO
DAMGO-Induced

Figure 5-4. M3G inhibition of evoked IPSCs in substantia gelatinosa neurons. IPSCs were evoked at a holding potential of 0 mV (K^+-gluconate-based internal solution in the patch pipette) by focal stimulation in the presence of 50 μM AP5 and 10 μM CNQX. A, a log-concentration response curve illustrating the concentration-dependent action of M3G on the IPSC. B, superimposed, averaged traces (n = 3) of evoked IPSCs before and during application of M3G (100 μM) (upper panel). Note that M3G strongly inhibits the IPSC. Superimposed, averaged traces (n = 3) of evoked IPSCs before and during application of morphine (300 nM) (lower panel). This low concentration of morphine was without effect. Traces shown in upper and lower panels were obtained from the same neuron. C, time course of changes in the amplitude of evoked IPSCs during application of morphine and M3G. D, superimposed, averaged traces (n = 6) of evoked IPSCs before and during application of M3G (100 μM) in the presence of naloxone (100 μM). E, time course of changes in the amplitude of evoked IPSCs during application of M3G and naloxone.

Figure 5-4

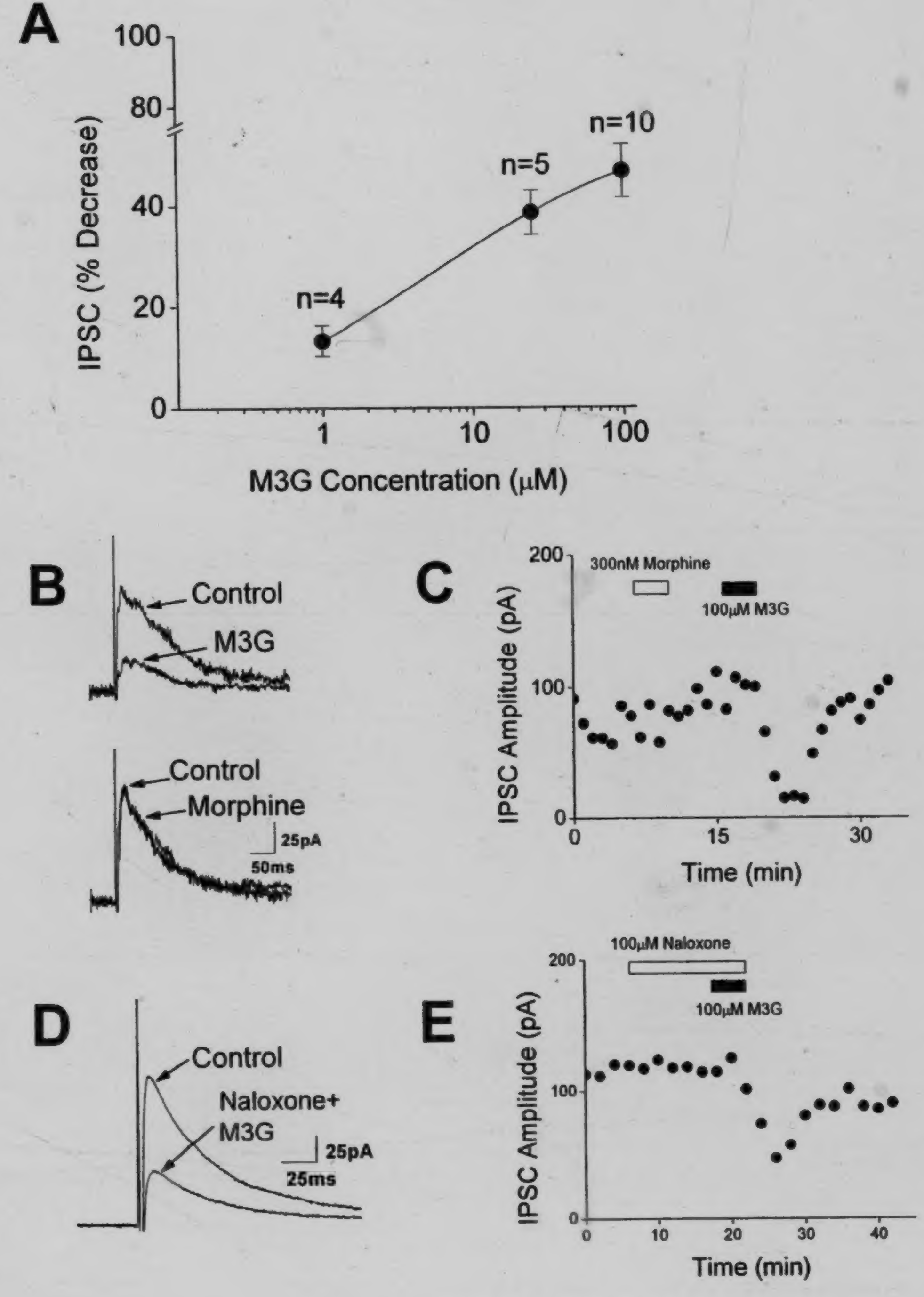

A
100
80
IPSC (% Decrease)
40
20
0
n=4
n=5
n=10
1
10
100
M3G Concentration (μM)
B
Control
M3G
Control
Morphine
25pA
50ms
C
200
100
0
300nM Morphine
100μM M3G
IPSC Amplitude (pA)
0
15
30
Time (min)
D
Control
Naloxone+
M3G
25pA
25ms
E
200
100
0
100μM Naloxone
100μM M3G
IPSC Amplitude (pA)
0
10
20
30
40
Time (min)

Figure 5-5. A, mIPSC traces before and during the application of M3G (100 μM). B and C, cumulative fraction plots of the mIPSC interevent interval and amplitude distribution. M3G significantly increased the interevent interval without affecting the amplitude distribution. The neuron was voltage clamped at a holding potential of 0 mV (K^+-gluconate-based internal solution in the patch pipette).

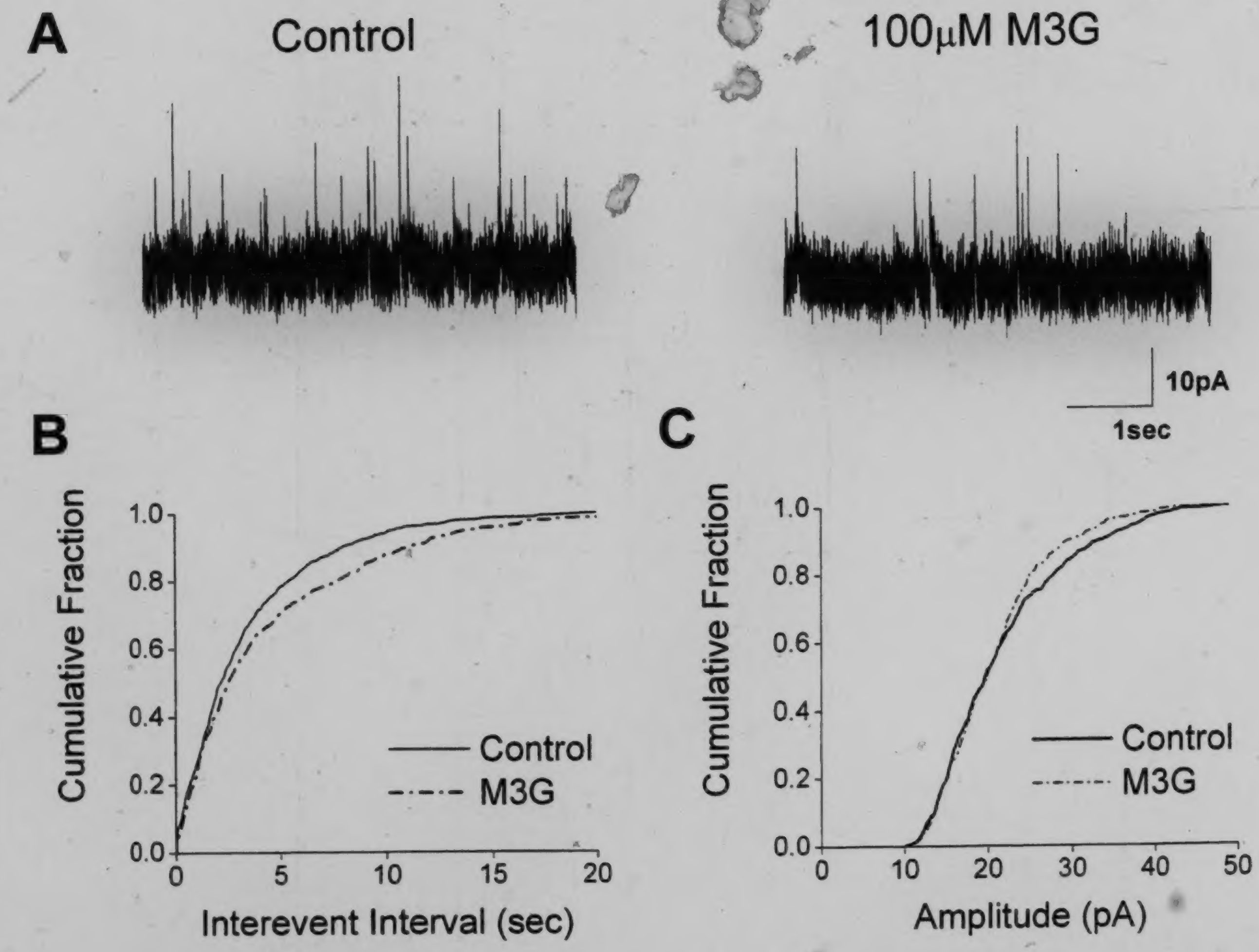

A
Control
100μM M3G
10pA
1sec
B
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Interevent Interval (sec)
Control
M3G
C
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Amplitude (pA)
Control
M3G

Figure 5-5

Figure 5-6. Suppression of the IPSC by M3G is accompanied by a change in the paired-pulse ratio. IPSCs were evoked at a holding potential of 0 mV (K^+-gluconate-based internal solution in the patch pipette) by focal stimulation in the presence of 50 μM AP5 and 10 μM CNQX. A, averaged traces (left panel; n = 10) of pairs of evoked IPSCs (100 ms inter-stimulus interval). M3G (100 μM) (right panel) reduces the amplitude of the evoked IPSCs. B, the change in the paired-pulse ratio caused by M3G is better observed after normalizing the data traces in A to the amplitude of the first IPSC. C, summary histogram of the change in paired-pulse ratio ($IPSC_2/IPSC_1$) induced by M3G (n = 4).

Figure 5-6

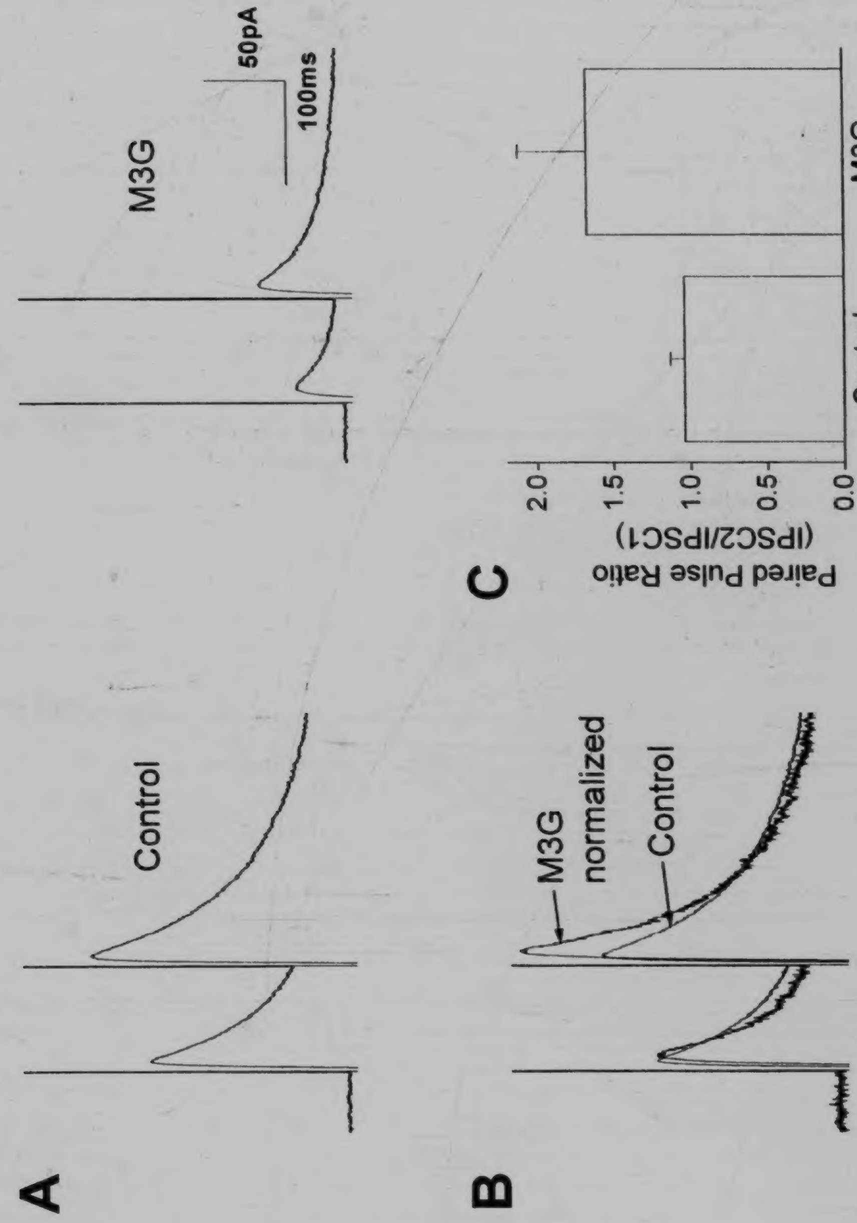

A
Control
M3G
50pA
100ms
B
M3G normalized
Control
C
Paired Pulse Ratio (IPSC2/IPSC1)
2.0
1.5
1.0
0.5
0.0
Control
M3G

Figure 5-7. M3G has similar actions on GABAergic and glycinergic synaptic transmission in substantia gelatinosa neurons. GABAergic IPSCs were evoked at a holding potential of 0 mV (K^+-gluconate-based internal solution in the patch pipette) by focal stimulation in the presence of 50 μM AP5, 10 μM CNQX and 1 μM strychnine. A, superimposed, averaged traces (n = 6) of evoked GABAergic IPSC before, during and after application of M3G (100 μM). Glycinergic IPSCs were evoked at a holding potential of 0 mV by focal stimulation in the presence of 50 μM AP5, 10 μM CNQX and 10 μM bicuculline. B, superimposed, averaged traces (n = 6) of evoked glycinergic IPSCs before, during and after application of M3G (100 μM). Note that both GABA- and glycine-mediated IPSCs are similarly affected by M3G. C and D, summary histograms of the effect of M3G on GABAergic (n = 4) and glycinergic IPSCs (n = 4).

Figure 5-7

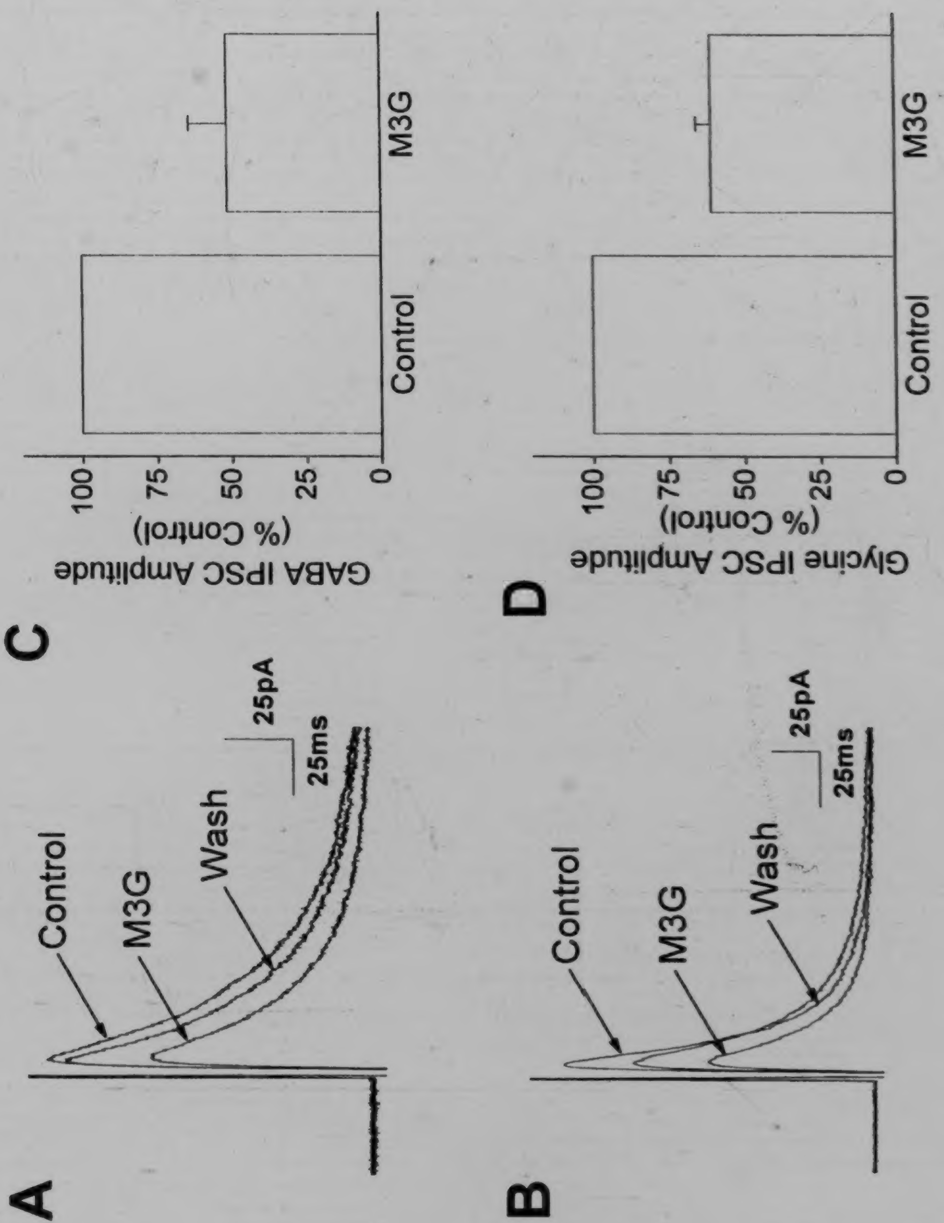
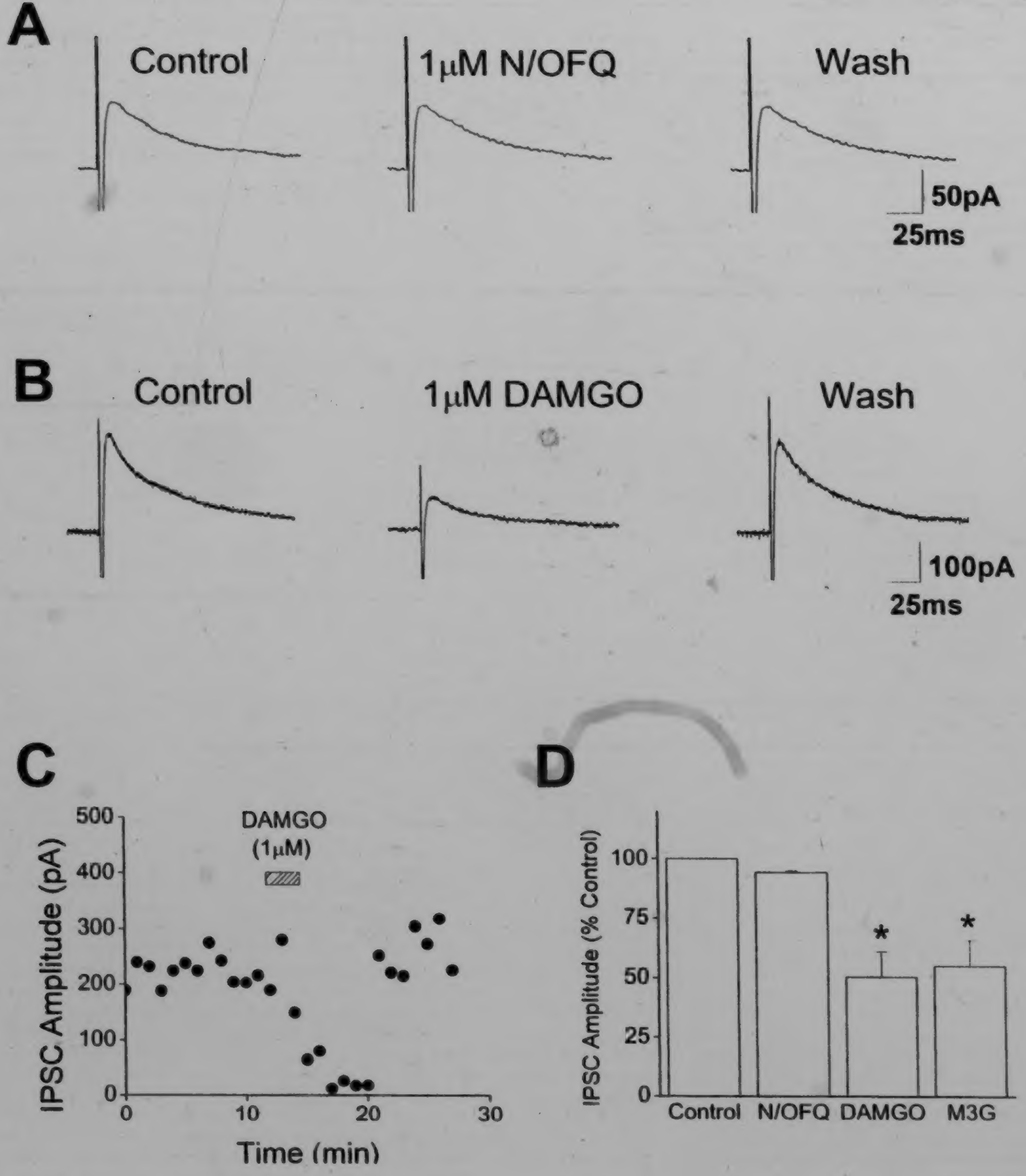

A
Control
M3G
Wash
25pA
25ms
B
Control
M3G
Wash
25pA
25ms
C
GABA IPSC Amplitude (% Control)
100
75
50
25
0
Control
M3G
D
Glycine IPSC Amplitude (% Control)
100
75
50
25
0
Control
M3G

Figure 5-8. Nociceptin/orphanin FQ (N/OFQ) and DAMGO have different actions on inhibitory synaptic transmission in substantia gelatinosa neurons. IPSCs were evoked at a holding potential of 0 mV (K^+-gluconate-based internal solution in the patch pipette) by focal stimulation in the presence of 50 μM AP5 and 10 μM CNQX. A, The upper panel shows averaged traces (n = 3) of evoked IPSCs before, during and after application of N/OFQ (1 μM). Note N/OFQ does not affect the amplitude of the evoked IPSC. B, averaged traces (n = 3) of evoked IPSCs before, during and after application of DAMGO (1 μM). Note that unlike N/OFQ, DAMGO reduces the amplitude of the evoked IPSC. C, time course of the amplitude of evoked IPSCs in the presence of DAMGO from the cell illustrated in B. D, summary histogram comparing the effects of N/OFQ (1 μM; n = 5), DAMGO, (1 μM; n = 6) and M3G (100 μM; n = 10) on IPSC amplitude (* = $p < 0.05$).

Figure 5-8


A
Control
1μM N/OFQ
Wash
50pA
25ms
B
Control
1μM DAMGO
Wash
100pA
25ms
C
DAMGO
(1μM)
IPSC Amplitude (pA)
500
400
300
200
100
0
0
10
20
30
Time (min)
D
IPSC Amplitude (% Control)
100
75
50
25
0
*
*
Control
N/OFQ
DAMGO
M3G

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Chapter 6

General Discussion

In this thesis, the cellular effects of NPY and M3G on substantia gelatinosa neurons were studied using an *in vitro* spinal cord slice preparation. These experiments are the first comprehensive electrophysiological studies of the actions of NPY and M3G in mammalian spinal cord dorsal horn. Moreover, as it has been generally regarded that obtaining stable electrophysiological recordings from spinal cord neurons is quite difficult, these experiments represent a significant achievement in advancing the understanding of the role of the substantia gelatinosa in pain modulation. This chapter will review the three preceding chapters. It will then address general issues regarding the actions of NPY and M3G in the dorsal horn and other regions of the CNS.

Chapter Summaries

Chapter 3

Chapter 3 described the electrophysiological properties of lamina II neurons in an adult rat spinal cord slice preparation developed to be used in conjunction with IR-DIC videomicroscopy. The combination of IR-DIC microscopy and patch-clamp recording had two clear advantages: (1) cells could be visually-identified and their viability could be assessed based on qualitative characteristics initially described by (Stuart *et al.*, 1993); and (2) patch-clamp recordings were made from the cell body and not other structures such as axons or dendrites, which may occur with 'blind' recordings (Blanton *et al.*, 1989). The experiments described in Chapter 3 also verified that the procedures used for spinal cord slice preparation yielded tissue that had similar pharmacological and synaptic properties to previous studies.

The first series of experiments demonstrated that primary afferent evoked EPSCs were mediated by glutamate. The application of CNQX and AP5 confirmed that EPSCs were mediated primarily by AMPA and NMDA receptors (Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1992; Yoshimura & Nishi, 1993). Activation of high-threshold primary afferents also generates EPSCs mediated in part by kainate receptors (Kerchner *et al.*, 2002; Li *et al.*, 1999). These receptors are present on primary afferent and interneuron terminals where they regulate glutamate, GABA and glycine release (Kerchner *et al.*, 2001a; Kerchner *et al.*, 2001b; Kerchner *et al.*, 2002). However, Chapter 3 and subsequent chapters did not address the relative contribution of kainate receptors to synaptic transmission in the dorsal horn.

Stimulation of local interneurons generated IPSCs that were mediated by GABA and glycine receptor activation. The GABAergic and glycinergic components were blocked by the $GABA_A$ receptor antagonist, bicuculline, and the glycine receptor antagonist, strychnine (Yoshimura & Nishi, 1993; Yoshimura & Nishi, 1995). Typically, the GABAergic IPSC was kinetically slower than the glycinergic IPSC, which has been described previously (Grudt & Henderson, 1998).

Because polysynaptic excitatory and inhibitory currents vary in amplitude and response latency (Yoshimura & Jessell, 1989; Yoshimura & Jessell, 1990; Yoshimura & Nishi, 1995) they are inappropriate for assessing the effects of neuromodulators on synaptic transmission. Thus, these experiments studied the effects on monosynaptic currents only. Monosynaptic currents were identified by their constant latency and reduced amplitude following high-frequency stimulation (10-20 Hz) (Hori *et al.*, 1996).

The experiments described in Chapter 3 were also undertaken to determine if GPCR signaling pathways were active and capable of modulating synaptic transmission in the substantia gelatinosa. This was assessed by application of the $GABA_B$ receptor agonist, baclofen. Baclofen (15-30 μM) potently suppressed excitatory and inhibitory synaptic transmission in lamina II and confirmed previous findings (Ataka *et al.*, 2000; Grudt & Henderson, 1998; Iyadomi *et al.*, 2000). In addition, these experiments were of particular significance as they established that GPCRs had not been compromised by the slice preparation procedures and that synaptic transmission could be modulated by agonists that act at GPCRs (e.g. μ-opioids, NPY).

Lastly, these experiments described procedures for *post-hoc* morphological identification of lamina II neurons. Biocytin labeling suggested that the majority of the neurons sampled were islet cells. This classification was based primarily on cell body shape, dendritic aborizations and location within lamina II (Bennett *et al.*, 1980; Gobel, 1978; Grudt & Perl, 2002; Spike & Todd, 1992; Woolf & Fitzgerald, 1983). However, this conclusion should be interpreted with caution because the dendrites of lamina II neurons extend rostro-caudally along the spinal cord and they may have been truncated by slicing in the transverse plane. Gobel and colleagues (Gobel, 1978; Gobel *et al.*, 1980) have suggested that stalked cells are the most abundant cell type in lamina II, but slicing in the transverse plane may produce a sampling bias towards islet cells (personal communication, A.B. MacDermott, Columbia University).

Chapter 4

Chapter 4 examined the pre- and postsynaptic actions of NPY on lamina II neurons. The principle findings were: (1) NPY suppressed excitatory synaptic transmission in the substantia gelatinosa via activation of a presynaptic Y2 receptor; (2) NPY suppressed GABAergic and glycinergic inhibitory synaptic transmission by pre- and postsynaptic Y1 receptors; (3) NPY activated an inwardly-rectifying conductance in about 25% of neurons sampled; and (4) NPY and μ-opioid receptors were frequently co-localized on the same presynaptic terminals, but infrequently co-localized on the postsynaptic membrane. The effects of NPY and μ-opioids on substantia gelatinosa neurons are summarized in Figure 6-1 and 6-2.

The finding that NPY suppressed EPSCs by a presynaptic Y2 receptor was supported by two lines of evidence. First, NPY affected the amplitude and the *ratio* of paired EPSCs. Second, NPY and the Y2-specific agonist, [Ahx$^{5\text{-}24}$]NPY, both reduced mEPSC frequency without affecting the amplitude distribution. This Y2 effect is consistent with previous binding and *in situ* hybridization studies (Mantyh *et al.*, 1994; Zhang *et al.*, 1995). These data were also supported by the ability of NPY and Y2 agonists to inhibit glutamate release from spinal cord synaptosomes (Martire *et al.*, 2000) and to suppress EPSPs in lamprey spinal cord (Parker *et al.*, 1998; Parker, 2000; Ullström *et al.*, 1999). Moreover, the presynaptic Y2 effect may explain the ability of NPY to reduce Substance P release from primary afferents innervating the substantia gelatinosa (Duggan *et al.*, 1991).

In DRG neuron somata, NPY suppresses N-type I_{Ca} and this effect is most pronounced in small diameter neurons (nociceptors) (Abdulla & Smith, 1999; Bleakman *et al.*, 1991; Walker *et al.*, 1988). It is therefore plausible that these receptors are transported to the central terminals where they couple to N-type Ca^{2+} channels and suppress transmitter release, which is similar to the mechanism by which NPY acts at sympathetic neuron terminals (Toth *et al.*, 1993). Because NPY and [Ahx^{5-24}]NPY affected mEPSCs, it is possible that Y2 agonists modulated synaptic release machinery at these terminals in addition to reducing transmitter release by an action on presynaptic Ca^{2+} channels. A similar mechanism for NPY has been shown in hypothalamic neurons (Rhim *et al.*, 1997; van den Pol *et al.*, 1996).

The effect of NPY on IPSCs is more complex. NPY or the Y1-specific agonist, [F7, P34]NPY, suppressed inhibitory synaptic transmission in lamina II and did so by acting at pre-and postsynaptic sites. Paired IPSCs indicated that NPY was acting at a presynaptic Y1 receptor. However, NPY and [F7, P34]NPY not only decreased mIPSC frequency, but also shifted the amplitude distributions to smaller values, suggesting involvement of a postsynaptic Y1 receptor. Both of these observations are consistent with Y1 receptor localization on presynaptic terminals (Bao *et al.*, 2002; Brumovsky *et al.*, 2002) and postsynaptic dendrites (Zhang *et al.*, 1994; Zhang *et al.*, 1999). Two mechanisms may account for the postsynaptic Y1 effect: (1) NPY or [F7, P34]NPY may have activated GIRK channels present in the dendrites and; (2) cross-talk may have occurred between the Y1 receptor and GABA and/or glycine receptors.

The effects of NPY were compared with the μ-opioid agonist, DAMGO. NPY and μ-opioid receptors were frequently co-localized on the same synaptic terminals as NPY and DAMGO potently suppressed excitatory synaptic transmission. The postsynaptic actions of NPY and DAMGO were similar, except NPY and μ-opioid receptors were almost never co-localized on the postsynaptic membrane. However, modulation of GIRK by NPY was observed too infrequently to determine the receptor subtype.

The presynaptic effect of NPY on excitatory transmission in the dorsal horn is reminiscent of its actions in hippocampus (Colmers *et al.*, 1987; Colmers *et al.*, 1988). In both dorsal horn and hippocampus, NPY acts via Y2 receptors to inhibit transmitter release. However, there are some differences between hippocampus and dorsal horn. In hippocampus, Y2 agonists affect sEPSCs, likely by inhibiting Ca^{2+} influx at the presynaptic terminal, but do not affect mEPSCs (McQuiston & Colmers, 1996), suggesting that NPY has additional presynaptic actions in the dorsal horn. The effect of NPY on mESPCs in the dorsal horn is similar to its actions in the arcuate nucleus and suprachiasmatic nucleus (SCN) of the hypothalamus (Rhim *et al.*, 1997; van den Pol *et al.*, 1996). In these regions, the effect of NPY is mediated via several presynaptic receptors, including Y1 and Y2 receptors. Additionally, the presynaptic effect of NPY in these regions was often long-lasting (> 30 min), as observed in the dorsal horn.

The actions of NPY on inhibitory synaptic transmission are similar to its effects in the paraventricular nucleus (PVN) (Pronchuk *et al.*, 2002) and SCN (Chen & van den Pol, 1996). In the PVN, multiple NPY receptors, including the Y1 receptor, inhibit

GABA-mediated IPSCs by an exclusively presynaptic mechanism. Similarly, in the SCN both Y1 and Y2, receptors inhibit GABAergic IPSCs and these receptors are co-expressed at pre- and postsynaptic sites (Chen & van den Pol, 1996). However, in thalamus Y1 and Y2 receptors appear to be functionally segregated; presynaptic Y2 receptors inhibit spontaneous and TTX-resistant GABAergic IPSCs (Sun *et al.*, 2001a), while postsynaptic Y1 receptors activate GIRK channels and Y2 receptors inhibit N- and P/Q-type Ca^{2+} channels in the soma and dendrites of thalamic neurons (Sun *et al.*, 2001b).

Chapter 5

Chapter 5 examined the effects of M3G on synaptic transmission in the substantia gelatinosa. Although the initial hypothesis that M3G was a putative ORL_1 agonist was rejected, new information about its actions in the dorsal horn was obtained. First, M3G had no effect on excitatory synaptic transmission and therefore did not act as an agonist at μ-opioid or ORL_1 receptors. Second, M3G did not antagonize the actions of DAMGO or N/OFQ on the EPSC. By contrast, the actions of M3G on inhibitory synaptic transmission were intriguing. Paired-pulse and mIPSC analysis indicated M3G selectively suppressed GABA and glycine via a presynaptic mechanism in all cells tested. The effects of M3G differed from NPY; M3G produced paired-pulse facilitation, while NPY produce paired-pulse depression. The reason for this difference may be due to different effects of each drug at the presynaptic terminal. Alternatively, it may simply be due to random response fluctuations in the paired pulse ratio, leading to spurious paired-pulse facilitation (Kim & Alger, 2001).

The effect of M3G was reminiscent of the actions of nocistatin in the dorsal horn (Ahmadi *et al.*, 2001; Zeilhofer *et al.*, 2000). It also provided a cellular mechanism to explain the hyperalgesia and allodynia that are produced following high-dose morphine administration (Woolf, 1981; Yaksh *et al.*, 1986; Yaksh & Harty, 1988). This study also showed μ-opioids suppressed GABA- and glycine-mediated IPSCs in the spinal cord dorsal horn, a finding that is controversial (see Kohno *et al.*, 1999).

Paradoxical Actions of NPY and μ-opioids: Comparing Behavioural and Cellular Experiments

Behavioural experiments have shown NPY is a potent analgesic (Broqua *et al.*, 1996; Hua *et al.*, 1991; Naveilhan *et al.*, 2001; Taiwo & Taylor, 2002) and this effect is mediated primarily by Y1 receptors (Broqua *et al.*, 1996; Naveilhan *et al.*, 2001; Taiwo & Taylor, 2002). However, the therapeutic potential of NPY for neuropathic pain treatment is complex (Hudspith & Munglani, 1999). In intact rats, intrathecal NPY had biphasic effects; at low doses, NPY enhanced the nociceptive flexor reflex, while higher doses caused long-lasting suppression of the reflex (Xu *et al.*, 1994). After axotomy, the biphasic effect of NPY remained, but the reflex depression caused by NPY occurred at concentrations lower than required for uninjured rats and was stronger and longer-lasting than in normal rats (Xu *et al.*, 1994). This biphasic action has been attributed to differential receptor activation. In intact rats, Y2 agonists cause reflex facilitation, but in axotomized animals Y2 agonists dose-dependently suppress the reflex (Xu *et al.*, 1999). By contrast, intrathecally-applied Y1 agonists caused reflex depression in both intact and axotomized rats. These findings suggest that Y1 and Y2 receptors differentially regulate nociceptive signaling in the dorsal horn. In addition, Y1 receptor knockout mice have increased pain behaviours in a variety of pain modalities, demonstrating the Y1 receptor has an essential anti-nociceptive role in pain signaling (Naveilhan *et al.*, 2001). Also, the observation that Y2 agonists begin to act as effective analgesics in neuropathic pain fits with the hypothesis of (Abdulla & Smith, 1999), that Y2 receptor up-regulation on DRG cell bodies may represent a model for a central action (e.g. Y2 modulation of transmitter release from primary afferent terminals) in neuropathic pain.

These behavioural experiments appear to present a paradox when interpreting the electrophysiological effects of NPY and μ-opioids on substantia gelatinosa neurons *in vitro*. In the present study, Y1 agonists selectively suppressed inhibitory synaptic transmission in the dorsal horn. Similarly, μ-opioids suppressed GABAergic and glycinergic transmission, consistent with previous findings (Grudt & Henderson, 1998). When viewed simplistically, these effects of Y1 agonists and μ-opioids would cause disinhibition in the substantia gelatinosa. This could result in increased synaptic activity and lead to the development of hyperalgesia and/or allodynia. Because Y1 agonists produce analgesia when administered intrathecally, it is possible that attenuation of inhibition contributes, paradoxically, to this effect. Although it is not intuitively obvious, disinhibition of a *few* neurons in a dominantly inhibitory network, such as the substantia gelatinosa (Beyer *et al.*, 1985; Sivilotti & Woolf, 1994; Yaksh, 1989; Zhang *et al.*, 2001), may lead to an increase in overall inhibitory tone, and hence an overall decrease in transmission of nociceptive information. By inference, μ-opioid suppression of GABAergic and glycinergic IPSCs in the substantia gelatinosa may produce analgesia via this mechanism. By contrast, M3G, which suppresses GABA and glycine release from *all* the neurons tested in the substantia gelatinosa (Moran & Smith, 2002), instead produces hyperalgesia and allodynia *in vivo*.

The experiments with NPY, μ-opioids and M3G demonstrated some of the inherent difficulties of comparing behavioural and cellular effects. They also drew attention to the importance of developing a comprehensive model of the neural circuitry of the substantia gelatinosa. Very few studies have made *exhaustive* correlations between the morphological and electrophysiological properties of superficial dorsal horn neurons (Grudt & Perl, 2002; Prescott & de Koninck, 2002) and no studies have correlated neurotransmitter/neuropeptide content with electrophysiological properties. However, a considerable amount of data is available regarding transmitter localization in dorsal horn neurons (Todd & Spike, 1993). Clearly, correlating the neurotransmitter phenotype of lamina II neurons with their electrophysiological and pharmacological responses could lead to better therapeutic interventions for neuropathic pain.

Future Directions

The effect of NPY in the spinal cord has provided a cellular basis for many of the analgesic effects of this neuropeptide. Because NPY has such potent analgesic effects in behavioural models of neuropathic pain, a very logical study would be to assess the effects of NPY in the dorsal horn of animals that have peripheral nerve injury. Because the effect of Y2 agonists becomes more pronounced in DRG neurons after nerve injury (Abdulla & Smith, 1999), it would be of interest to determine if the effects of Y2 agonists are enhanced in the dorsal horn following peripheral nerve injury.

The cellular effects of NPY on synaptic transmission in the substantia gelatinosa suggest NPY may have important roles in other areas responsible for modulating pain transmission. Behavioural studies indicate that NPY administered into the PAG (Glaum *et al.*, 1994; Nothacker *et al.*, 1996) and the nucleus accumbens (Yajiri *et al.*, 1997) contributes to anti-nociceptive effects of NPY. Since a great deal is known about the synaptic circuitry and the effects of opioids in these regions (Brundege & Williams, 2002; Chieng & Williams, 1998; Connor & Christie, 1998; Osborne *et al.*, 1996; Vaughan & Christie, 1997) studying the effects of NPY on neurons in these regions represents a logical extension of the present study.

If NPY were to be an effective treatment for neuropathic pain, non-peptide Y1 and Y2 agonists would have to be developed. Although it is relatively easy to use chemical modeling techniques to design compounds that will selectively bind to Y1 and Y2 receptors (or other peptide receptors), such compounds usually exhibit antagonist properties. However, this search is not futile, as non-peptide agonists for peptide receptors clearly exist in nature, morphine being the prime example.

Figure 6-1. Diagram illustrating the neurophysiological actions of neuropeptide Y on excitatory synaptic transmission in the substantia gelatinosa. NPY suppresses glutamate release by acting at presynaptic Y2 receptors. AMPA, kainate and NMDA receptors are shown on the postsynaptic neuron. Legend: $GABA_BR$ ($GABA_B$ receptor); VDCC (voltage-dependent calcium channel); μ-opioid R (μ-opioid receptor); Y2 (NPY Y2 receptor).

Figure 6-1

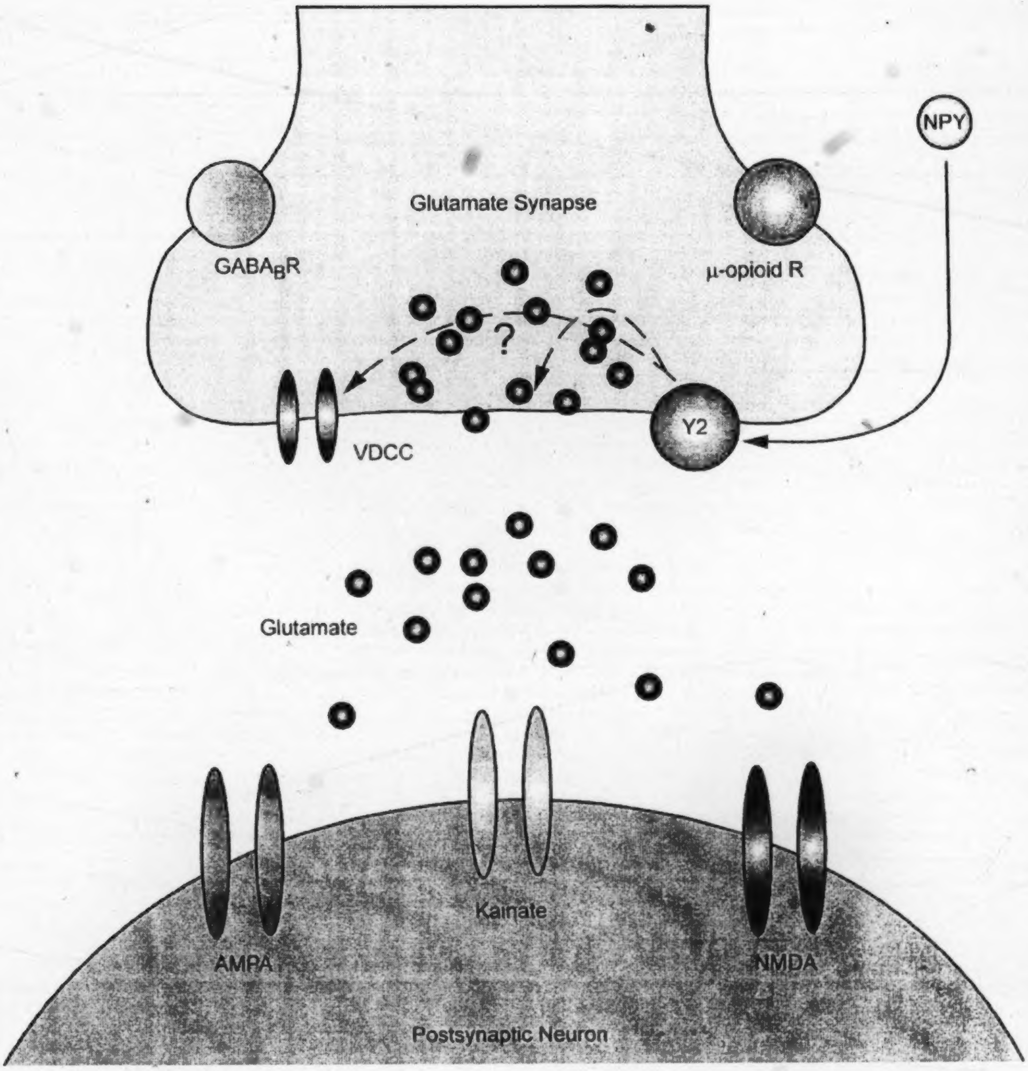
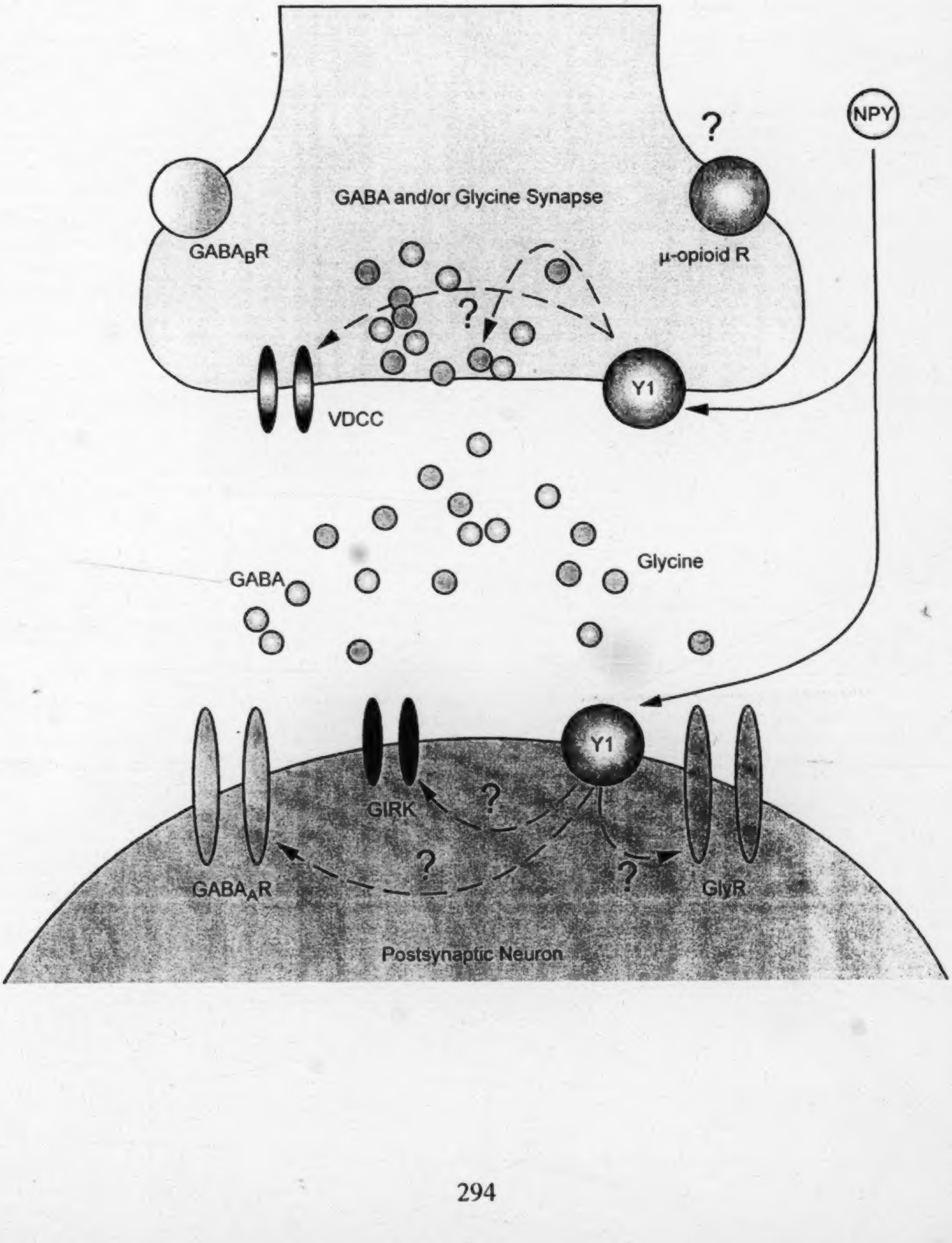

NPY
Glutamate Synapse
$GABA_BR$
μ-opioid R
?
VDCC
Y2
Glutamate
AMPA
Kainate
NMDA
Postsynaptic Neuron

Figure 6-2. Diagram illustrating the neurophysiological actions of neuropeptide Y on inhibitory synaptic transmission in the substantia gelatinosa. NPY suppresses GABA and glycine release by acting at presynaptic Y1 receptors. Y1 receptors are also located on the postsynaptic neuron and may directly modulate GABA and/or glycine receptors. NPY also activates an inwardly-rectifying conductance, presumably a GIRK channel. Legend: $GABA_B$ R ($GABA_B$ receptor); $GABA_A$R ($GABA_A$ receptor); GlyR (glycine receptor); μ-opioid R (μ-opioid receptor); GIRK (G-protein-coupled inwardly-rectifying K^+ channel); VDCC (voltage-dependent calcium channel); Y1 (NPY Y1 receptor).

Figure 6-2


GABA and/or Glycine Synapse
NPY
?
$GABA_BR$
μ-opioid R
?
VDCC
Y1
GABA
Glycine
Y1
GIRK
?
?
?
$GABA_AR$
GlyR
Postsynaptic Neuron

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